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THE PRINCIPLES

OF

BACTERIOLOGY:

A PRACTICAL MANUAL FOR STUDENTS AND PHYSICIANS.

BY

A. C. ABBOTT, M. D.,

PROFESSOR OF HYGIENE, AND DIRECTOR OF THE LABORATORY OF HYGIENE, UNIVERSITY OF PENNSYLVANIA.

FIFTH EDITION,

ENLARGED AND THOROUGHLY REVISED.

With 109 Illustrations, of which 26 are colored.

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PREFACE TO THE FIFTH EDITION.

THE call for the fifth edition of this book within two years of the publication of the fourth is naturally a source of very great gratification to the author, and he takes this occasion to express his obligations to those of his readers who have materially contributed to the success of the work by friendly comments and criticism.

The plan of this edition does not differ from that followed in its predecessors. Reference has been made to such investigations of the past two years as are pertinent to a book of this character, and essential to an understanding on the part of the reader of the lines along which the subject has advanced. Several chapters, particularly those on technique, disinfection, the specific infections, and immunity have been considerably enlarged. In some cases opinions expressed in former editions have been modified to conform to more advanced views. A number of the old illustrations have been replaced by new ones, several of which are in colors. The author trusts that such alterations as have been made will be regarded as adding to the value of the work.

PHILADELPHIA, March, 1899.

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PREFACE TO THE FIRST EDITION.

In preparing this book the author has kept in mind the needs of the student and practitioner of medicine, for whom the importance of an acquaintance with practical bacteriology cannot be overestimated.

It is to advances made through bacteriological research that we are indebted for much of our knowledge of the conditions underlying infection, and for the elucidation of many hitherto obscure problems concerning the etiology, the modes of transmission, and the means of prevention of infectious maladies.

Only within a comparatively short time have students and physicians been enabled to obtain the systematic instruction in this science that is of value in aiding them in their efforts to check disease. The rapid increase in the number who are availing themselves of these opportunities speaks directly for the practical value of the science.

As the majority of those undertaking the study of bacteriology do so with the view of utilizing it in medical practice, and as many of these can devote to it but ortion of their time, it is desirable that the subject- r be presented in as direct a manner as possible.

Presuming the reader to be unfamiliar with the subject, the author has restricted himself to those fundamental features that are essential to its understanding. The object has been to present the important ideas and methods as concisely as is compatible with clearness, and at the same time to accentuate throughout the underlying principles which govern the work.

With the view of inducing independent thought on the part of the student, and of diminishing the frequency of that oft-heard query, "What shall I do next?" experiments have been suggested wherever it is possible. These have been arranged to illustrate the salient points of the work and to attract attention to the minute details, upon the observation of which so much in bacteriology depends.

PHILADELPHIA, December, 1891.

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Though the contributions which have done most to place bacteriology on the footing of a science are those of recent years, still, during the earlier stages of its development, many observations were made which formed the foundation-work for much that was to follow. Before regularly beginning our studies, therefore, it may be of advantage to acquaint ourselves with the more prominent of these investigations.

Antony van Leeuwenhoek, the first to describe the bodies now recognized as bacteria, was born at Delft, in Holland, in 1632. He was not considered a man of liberal education, having been during his early years an apprentice to a linendraper. During his apprenticeship he learned the art of lens-grinding, in which he became so proficient that he eventually perfected a simple lens by means of which he was enabled to see objects of much smaller dimensions than any hitherto seen with the best compound microscopes in existence at that date. At the time of his discoveries he was following the trade of linendraper in Amsterdam.

In 1675 he published the fact that he had succeeded in perfecting a lens by means of which he could detect in a drop of rain-water living, motile "animalcules" of the most minute dimensions—smaller than anything that had hitherto been seen. Encouraged by this discovery, he continued to examine various substances for the presence of what he considered animal life in its most minute form. He found in sea-water, in well-water, in the intestinal canal of frogs and birds, and in his own diarrhœal evacuations, objects that differentiated themselves the one from the other, not only by their shape and size, but also by the peculiarity of motility which some of them were seen to possess.

In the year 1683 he discovered in the tartar scraped from between the teeth a form of micro-organism upon which he laid special stress. This observation he embodied in the form of a contribution which was presented to the Royal Society of London on September 14, 1683. This paper is of particular importance, not only because of the careful, objective nature of the description given of the bodies seen by him, but also for the illustrations which accompany it. From a perusal of the text and an inspection of the plates accompanying it there remains little room for doubt that Leeuwenhoek saw with his primitive lens the bodies now recognized as bacteria.[1]

Upon seeing these bodies he was apparently very much impressed, for he writes: "With the greatest astonishment I observed that everywhere throughout the material which I was examining were distributed animalcules of the most microscopic dimensions, which moved themselves about in a remarkably energetic way."

This discovery was shortly followed by others of an equally important nature. His field of observation appears to have increased rapidly, for after a time he speaks of bodies of much smaller dimensions than those at first described by him.

Throughout all of Leeuwenhoek's work there is a conspicuous absence of the speculative. His contributions are remarkable for their purely objective nature.

After the presence of these organisms in water, in the mouth, and in the intestinal evacuations was made known to the world, it is hardly surprising that they were immediately seized upon as the explanation of the

[1] See Arcana Naturæ detecta ab ANTONIO VAN LEEUWENHOEK; Delphis Batavorum, 1695.

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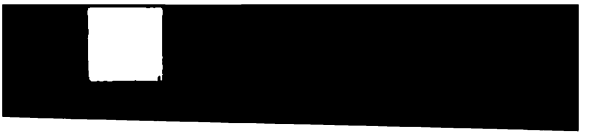
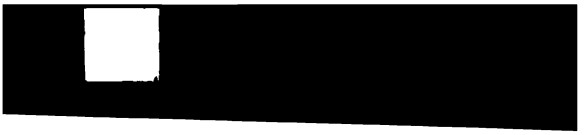


by Treviranus, viz., that the high temperature to which the infusions had been subjected had so altered them and the air about them that the conditions favorable to spontaneous generation no longer existed, was promptly met by Spallanzani, when he gently tapped one of the flasks that had been boiled against a hard object until a minute crack was produced; invariably organisms and decomposition appeared in the flask thus treated.

From the time of the experiments of Spallanzani until as late as 1836 but little advance was made in the elucidation of this obscure problem.

In 1836 Schulze attracted attention to the subject by the convincing nature of his investigations. He showed that if the air which gained access to boiled infusions was robbed of its living organisms by being caused to pass through strong acid or alkaline solutions no decomposition occurred, and living organisms could not be detected in the infusions. Following quickly upon this contribution came Schwann, in 1837, and somewhat later (1854) Schröder and Dusch, with similar results obtained by somewhat different means. Schwann deprived the air which passed to his infusions of its living particles by conducting it through highly heated tubes; whereas Schröder and Dusch, by means of cotton-wool interposed between the boiled infusions and the outside air, robbed the air passing to the infusions of its organisms by the simple process of filtration. In 1860 Hoffmann and in 1861 Chevreul and Pasteur demonstrated that the precautions taken by preceding investigators for rendering the air which entered these flasks free from bacteria were not necessary; that all that was necessary to prevent the access of bacteria to the infusions in the flasks was to draw out the neck of the flask



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to further investigations, and as the all-important question was that concerning the relation of these microscopic organisms to disease, attention naturally turned into this channel of study. Even before the hypothesis of spontaneous generation had received its final refutation a number of observations of a most important nature had been made by investigators who had long since ceased to consider spontaneous generation as a tenable explanation of the origin of the microscopic living particles.

In the main, these studies had been conducted upon wounds and the infections to which they are liable; in fact, the evolution of our knowledge of bacteriology to its present development is so intimately associated with this particular line of investigation that a few historical facts in connection with it may not be without interest.

The observations of Rindfleisch, in 1866, in which he describes the presence of small, pin-head points in the myocardium and general musculature of individuals that had died as a result of infected wounds, represent, probably, the first reliable contribution to this subject. He studied the tissue-changes round about these points up to the stage of miliary abscess-formation. He refers to the organisms as "vibrios." Almost simultaneously von Recklinghausen and Waldeyer described similar changes that they had observed in pyæmia and, occasionally, secondary to typhoid fever. Von Recklinghausen believed the granules seen in the abscess-points to be micrococci and not tissue-detritus, and gave as the reason that they were regular in size and shape, and gave specific reactions with particular staining-fluids. Birch-Hirschfeld was able to trace bacteria found in the blood and organs to the wound as the point of entrance, and believed both the local and the constitutional

condition to stand in direct ratio to the number of spherical bacteria present in the wound. He observed also that as the organisms increased in number they could often be found within the bodies of pus-corpuscles. His studies of pyæmia led him to the important conclusion that in this condition micro-organisms were *always* present in the blood.

Of immense importance to the subject were the investigations of Klebs, made at the Military Hospital at Carlsruhe in 1870–'71. He not only saw, as others before him had seen, that bacteria were present in diseases following infection of wounds, but described the manner in which the organisms had gained entrance from the point of injury to the internal organs and blood. He expressed the opinion that the spherical and rod-shaped bodies which he saw in the secretions of wounds were closely allied, and he gave to them the designation "microsporon septicum." He believed that the organisms gained access to the tissues round about the point of injury both by the aid of the wandering leucocytes and by being forced through the connective-tissue lymph-spaces by the mechanical pressure of muscular contraction.

On erysipelatous inflammations secondary to injury important investigations were also being made, Wilde, Orth, von Recklinghausen, Lukomsky, Billroth, Ehrlich, Fehleisen, and others agreeing that in these conditions micro-organisms could always be detected in the lymph-channels of the subcutaneous tissues; and through the work of Oertel, Nassiloff, Classen, Letzerich, Klebs, and Eberth the constant presence of bacteria in the diphtheritic deposits at times seen on open wounds was established.

Simple and natural as all this may seem to us now, the stage to which the subject had developed when these observations were recorded did not admit of their meeting with unconditional acceptance. The only strong argument in favor of the etiological relation of the organisms that had been seen to the diseases with which they were associated was the constancy of this association. No efforts had been made to isolate them, and few or none to reproduce the pathological conditions by inoculation. Moreover, not a small number of investigators were skeptical as to the importance of these observations; many claimed that micro-organisms were normally present in the blood and tissues of the body; and some even urged that the organisms seen in diseased conditions were the result rather than the cause of the maladies. It is hardly necessary to do more than say that both of these views were purely speculative, and have never had a single reliable experimental argument in their favor. Billroth and Tiegel, who held to the former opinion, did endeavor to prove their position through experimental means; but the methods employed by them were of such an untrustworthy nature that the fallacy of deductions drawn from them was very quickly demonstrated by subsequent investigators. Their method for demonstrating the presence of micro-organisms in normal tissues was to remove bits of organs from the healthy animal body with heated instruments and drop them into hot melted paraffin, they holding that all living organisms on the surface of the tissues would be destroyed by the high temperature, and that if decomposition should subsequently occur it would prove that it was the result of the growth of bacteria in the depths of the tissues to which the heat had not penetrated.

Decomposition did usually set in, and they accepted this as proof of the accuracy of their view. Attention was, however, shortly called to the fact that in cooling there was contraction of the paraffin, resulting usually in the production of small rents and cracks in which dust, and bacteria lodged upon it, could accumulate and finally gain access to the tissues, with the occurrence of decomposition as a consequence. Their results were thus explained after a manner analogous to that employed by Spallanzani, in 1769, in demonstrating to Treviranus the fallacy of the opinion held by him and the accuracy of his own views, viz., that it was always through the access of organisms from without that decomposition primarily originated. (See page 23.)

Under careful precautions, against which no objection could be raised, the experiments of Billroth and Tiegel were repeated by Pasteur, Burdon-Sanderson, and Klebs, but with failure in every instance to demonstrate the presence of bacteria in the healthy living tissues.

The fundamental researches of Koch (1881) upon pathogenic bacteria and their relation to the infectious diseases of animals differed from those of preceding investigators in many important respects. The scientific methods of analysis with which each and every obscure problem was met as it arose served at once to distinguish him as a pioneer in this hitherto but imperfectly cultivated domain. The outcome of these investigations was the establishment of a foundation upon which the bacteriology of the future was to rest. He, for the first time, demonstrated that distinct varieties of infection, as evidenced by anatomical changes, are due in many cases to the activities of specific micro-organisms, and that by proper methods it is possible to isolate these

organisms in pure culture, to cultivate them indefinitely, to reproduce the conditions by inoculation of these pure cultures into susceptible animals, and to continue the disease at will by continuous inoculation from an infected to a healthy animal. By the methods that he employed he demonstrated a series of separate and distinct diseases that can be produced in mice and rabbits by the injection of putrid substances into their tissues. The disease known as septicæmia of mice, likewise a disease characterized by progressive abscess-formation, and pyæmia and septicæmia of rabbits, were among the affections produced by him in this way. It was in the course of this work that the Abbe system of substage condensing apparatus was first used in bacteriology; that the aniline dyes suggested by Weigert were brought into general use; that the isolation and cultivation of bacteria in pure culture on solid media were shown to be possible; and that animals were employed as a means of obtaining from mixtures pure cultures of pathogenic bacteria.

With the bounteous harvest of original and important suggestions that was reaped from Koch's classical series of investigations bacteriology reached an epoch in its development, and at this period modern bacteriology may justly be said to have had its birth.

NOTE.—I have presented only the most prominent investigations that will serve to indicate the lines along which the subject has developed. For a more detailed account of the historical development of the work the reader is referred to Löffler's instructive and entertaining *Vorlesungen über die geschichtliche Entwickelung der Lehre von den Bacterien*, upon which I have drawn largely in preparing the foregoing sketch.

CHAPTER I.

Definition of bacteria—Their place in nature—Differences between parasites and saprophytes—Nutrition of bacteria—Products of bacteria—Their relation to oxygen—Influence of temperature upon their growth.

By the term bacteria is understood that large group of minute vegetable organisms the individual members of which multiply by a process of transverse division. They are spherical, oval, rod-like, and spiral in shape, and are commonly devoid of chlorophyll.[1] Owing to the absence of chlorophyll from their composition bacteria are forced to obtain their nutritive materials from organic matters as such, and lead, therefore, either a saprophytic[2] or parasitic[3] existence.

Their life-processes are so rapid, complex, and energetic that they result in the most profound alterations in the structure and composition of the materials in and upon which they are developing.

Decomposition, putrefaction, and fermentation result

[1] Chlorophyll is the green coloring-matter possessed by the higher plants by means of which they are enabled in the presence of sunlight to decompose carbonic acid (CO_2) and ammonia (NH_3) into their elementary constituents.

[2] A saprophyte is an organism that obtains its nutrition from dead organic matter.

[3] A parasite lives always at the expense of some other living, organic creature, known as its host, and in the strictest sense of the word cannot develop upon dead matter. There is, however, a group of so-called "facultative" saprophytes and parasites which possess the power of accommodating themselves to existing surroundings—at one time leading a parasitic, at another time a saprophytic existence.

from the activities of the *saprophytic* bacteria; while the changes brought about in the tissues of their host by the purely *parasitic* forms find expression in disease-processes and not infrequently in complete death.

The rôle played in nature by the saprophytic bacteria is a very important one. Through their functional activities the highly complicated tissues of dead animals and vegetables are resolved into the simpler compounds, carbonic acid, water, and ammonia, in which form they may be taken up and appropriated as nourishment by the more highly organized members of the vegetable kingdom. It is through this ultimate production of carbonic acid, ammonia, and water by bacteria, as end-products in the processes of decomposition and fermentation of dead animal and vegetable tissues, that the demands of growing vegetation for these compounds are supplied.

The chlorophyll plants do not possess the power of obtaining their carbon and nitrogen from such highly organized and complicated substances as serve for the nutrition of bacteria, and as the production of the simpler compounds, carbon dioxide and ammonia, by the animal world is not sufficient to meet the demands of the chlorophyll plants, the importance of the part played by bacteria in making up this deficit cannot be overestimated. Were it not for the activity of these microscopic living creatures all life upon the surface of the earth would cease. Deprive higher vegetation of the carbon and nitrogen supplied to it as a result of bacterial activity, and its development comes rapidly to an end; rob the animal kingdom of the food-stuffs supplied to it by the vegetable world, and life is no longer possible. It is plain, therefore, that the saprophytes, which represent the large majority of all bacteria, must be

looked upon in the light of benefactors, without which existence would be impossible.

With the parasites, on the other hand, the conditions are far from analogous. Through their metabolic activities there is constantly a loss, rather than a gain, to both the animal and vegetable kingdoms. Their host must always be a living body in which exist conditions favorable to their development, and from which they appropriate substances that are necessary to the health and life of the organism to which they have found access; at the same time they eliminate substances as products of their nutrition that are directly poisonous to the tissues in which they are growing.

In their relations to terrestrial life, the positions occupied by the two functionally different groups, the saprophytes on the one hand, and the parasites on the other, are diametrically opposite: the saprophytic forms stand in the relation of benefactors, in resolving dead animal and vegetable bodies into their component parts, which serve as food for living vegetation, and at the same time they remove from the surface of the earth the remains of all dead organic substances; while the parasitic group exists only at the expense of the more highly organized members of both the animal and vegetable kingdoms. It is to the parasitic group that the *pathogenic*[1] organisms belong.

In addition to the saprophytes that are concerned in the changes to which allusion has just been made, there exist other saprophytic forms whose life-processes result in specific changes of most interesting and important natures. Some of these are characterized by their prop-

[1] Pathogenic organisms are those which possess the property of producing disease.

erty of producing pigments of different color; these are known as the *chromogenic*[1] forms. Just what is their exact rôle in nature it is difficult to say; but it is probable that, in addition to their most conspicuous function of color-production, they are also in some way concerned in the omnipresent process of disintegration which is constantly going on in all dead organic substances. From the investigations of Beyerinck it would seem that in some of these chromogenic forms the pigment is an integral part of the bacteria themselves; in others that it is an excretory product of species that are themselves colorless; while in still others, that it is an excretory product which remains intimately associated with the bacterial cells and in part or wholly stains them.

Others, the so-called *photogenic* or phosphorescent bacteria, possess the property of producing light or of illuminating the medium on which they grow by a peculiar phosphorescence. These are found in sea-water and in decomposing phosphorescent fish and meat.

Still others, the so-called *zymogenic* bacteria, are concerned in the various fermentations, such, for instance, as acetic-, lactic-, and butyric-acid fermentations; and many of the industries, such, for example, as those concerned in the making of wine, beer, cheese, butter, and indigo, are more or less directly dependent upon the fermentation that accompanies the growth of peculiar species of bacteria in those materials.

Other species of bacteria liberate in the course of their growth soluble proteolytic ferments or enzymes, more or less analogous to pepsin and trypsin in their

[1] Chromogenic: possessing the property of producing color.

action upon proteids; while others generate ferments analogous to diastase and to invertin: the former converting starch into sugar, and the latter having the power of converting cane- into grape-sugar.

The *saprogenic* bacteria are those that produce the particular fermentation that we know as putrefaction.

Another very important saprophytic group comprises the so-called *nitrifying* and *denitrifying* bacteria, whose activities are concerned in specific forms of fermentation: the former oxidizing ammonia to nitrous and nitric acids; the latter reducing nitric acid to nitrous acid and ammonia. It is through their association (symbiosis) with the nitrifying bacteria that certain plants, the leguminous, are enabled to make up their nitrogen deficit in part from the free nitrogen of the air. The discovery of this phenomenon gave to free atmospheric nitrogen a biological significance that had hitherto been denied it.

The so-called *thiogenic* bacteria convert sulphuretted hydrogen into higher sulphur compounds.

The progress of many forms of fermentation and decomposition is accompanied by more or less elevation of temperature. This is seen in the curing of manure, tobacco, malt, etc.

As stated, bacteria that produce disease are known as *pathogenic*. They induce disease by their poisonous action upon the tissues in which they are located. The materials of which certain species of bacteria are constructed, and the products of growth of certain others, are of the greatest importance in their relation to animal and human pathology. Particular species, while not eliming soluble poisons as a product of metabolism, are

nevertheless themselves built up of poisonous proteids, or of proteids with which toxic materials are so intimately associated that they can only be isolated by the most refined and elaborate chemical manipulations. Others produce in the course of their growth soluble poisons that can readily be separated by very simple methods from the bacteria that produced them. The proteid matters making up the bodies of many species of bacteria, even those not conspicuously pathogenic, have been shown by Buchner to induce disease when isolated and injected into the tissues of animals; in some cases causing only rise of body-temperature, in others acute inflammatory processes with pus-formation. To such proteids Buchner has given the name *bacterial proteins.*

The poisonous soluble products of bacterial growth are severally known as *toxins, toxalbumins,* and *ptomains:* toxins being, in general, uncrystallizable poisons of whose intimate chemical nature little or nothing is known; toxalbumins being those that partake of the chemical nature of albumins; while ptomains are crystallizable products of bacterial activity which, physically speaking, are analogous to the ordinary vegetable alkaloids.

We have said that through the agency of chlorophyll, in the presence of sunlight, the green plants are enabled to obtain the amount of nitrogen and carbon which is necessary to their growth from such simple bodies as carbon dioxide and ammonia, which they decompose into their elementary constituents. The bacteria, on the other hand, owing to the absence of chlorophyll from their tissues, do not possess this power. They must, therefore, have their carbon and nitrogen pre-

sented as such, in the form of decomposable organic substances.

In general, bacteria obtain their nitrogen most readily from soluble albumins, and to a certain extent, but by no means so easily, from salts of ammonium. In some of Nägeli's experiments it appeared probable that they could obtain the necessary amount of nitrogen from inorganic nitrates. At all events, he was able in certain cases to demonstrate a reduction of nitric to nitrous acid and ultimately to ammonia. Nevertheless, in all of these experiments circumstances point to the probability that the nitrogen obtained by the bacteria for building up their tissues in the course of their development was derived from some source other than the nitric acid or the nitrates, and that the reduction of this acid was most probably a secondary phenomenon. It must be borne in mind, however, that there exists a specific group of bacteria, the nitrifying bacteria, that apparently increase and multiply without appropriating proteid nutrition. They are concerned in the particular form of fermentation that results in the oxidation of ammonia to nitrous and nitric acids, a process everywhere in progress in the superficial layers of the soil.

For the supply of carbon many of the carbon compounds serve as sources upon which the bacteria can draw. The carbon deficit, for example, can be obtained from sugar and bodies of like composition; from glycerin and many of the fatty acids; and from the alkaline salts of tartaric, citric, malic, lactic, and acetic acids. In some instances carbon compounds, which when present in concentrated form inhibit the growth of bacteria, may, when highly diluted, serve as nutri-

tion for them. Salicylic acid and ethyl alcohol are of this class.

In addition to carbon and nitrogen, water is essential to the life and development of bacteria. Without it no development occurs, and in many cases drying the organisms results in their death. Certain species and developmental forms, on the contrary, though incapable of multiplying when in the dry state, may be completely deprived of their water without causing them to lose the power of reproduction under favorable conditions.

Closer study of bacteria, and a more intimate acquaintance with their nutritive changes, demonstrate an appreciable variability in the character of the substances best suited for the nutrition of different species; one requiring a tolerably concentrated form of nutrition, while another needs but a very limited amount of proteid substance for its development. Certain of them bring about most profound alterations in the media in which they exist, while others produce but little apparent change. In one case alterations in the reaction of the media will be conspicuous, while in another no such variation can be detected. With the growth of some forms products resulting from specific processes of fermentation appear. Other varieties produce poisons of remarkable degrees of toxicity, while the growth of others may be accompanied by the evolution of compounds characteristic of putrefaction.

In considering the normal development of bacteria we must not lose sight of the fact that this is influenced both by the quality and the quantity of the nutritive materials to which they have access, and by the character of the products that accumulate in these materials as a

result of their vital processes. Nitrogen and carbon compounds may be present in amount and kind entirely suitable to normal bacterial growth, and yet this may be checked, after a comparatively short time, by the accumulated products of bacterial metabolism, some of which possess the property of inhibiting growth and ultimately of even destroying the bacteria. The most common and conspicuous examples of the inhibiting conditions that are coincident with bacterial growth are alterations in the chemical reaction of the matters in which the bacteria are developing.

Since the majority of them grow best in media of a neutral or very slightly alkaline reaction, any excessive development of alkalinity or acidity, as a result of growth, arrests development, and no evidence of life or further multiplication can be detected until this deviation from the neutral reaction has been corrected.

Of considerable importance and interest in the study of the nutritive changes of bacteria is the difference in their relation to oxygen. For certain species free oxygen is essential to the proper performance of their functions; in another group no evidence of life can be detected under its access; while in a third group oxygen appears to play but an unimportant rôle, for development occurs as well with as without it. It was Pasteur who first demonstrated the existence of particular species of bacteria which not only grow and multiply and perform definite physiological functions without the aid of free oxygen, but to the existence of which it is positively harmful. To these he gave the name *anaërobic* bacteria, in contradistinction to the *aërobic* group, for the proper performance of whose functions

oxygen is essential. In addition to these there is a third group, for the maintenance of whose existence the absence or presence of oxygen is apparently of no moment—development progresses as well with as without it; the members of this group comprise the class known as *facultative* in their relation to this gas. It is to this third group, the facultative, that the majority of bacteria belong. Since all growing bacteria, anaërobic as well as aërobic, generate carbonic acid in the course of their development, it is evident that oxygen must in reality be obtained by them from some source, and must be regarded as essential to their life-processes; but the manner in which it is appropriated by them varies, the aërobic species taking it from the air as free oxygen, while the anaërobic species, not possessed of this power, obtain it through the decomposition of more or less stable oxygen-containing compounds.

Though the multiplication of the facultative varieties is not interfered with by either the presence or absence of free oxygen, yet experiments demonstrate that the products of their growth are different under the varying conditions of absence or presence of this gas. For example: in the case of certain of the chromogenic forms the presence or absence of oxygen has a very decided effect upon the production of the pigments by which they are characterized.

NOTE. Observe the difference between the intensity of color produced upon the surface of the medium and that along the track of the needle in stab-cultures of *bacillus prodigiosus* and of *spirillum rubrum*. In the former the red color is apparently a product dependent upon the presence of oxygen, while in the latter the

greatest intensity of color occurs at the point furthest removed from the action of oxygen.

Another factor which plays a highly important part in the biological functions of these organisms is the temperature under which they exist. The extremes of temperature between which the majority of bacteria are known to grow range from 5.5° to 43° C. At the former temperature development is hardly appreciable; it becomes more and more active until 38° C. is reached, when it is at its optimum, and, as a rule, ceases at 43° C.; though species exist that multiply at as high a temperature as 70° C. and others at as low as 0° C. The studies of Globig,[1] Miquel,[2] and Macfadyen and Bloxall[3] have demonstrated that there exist in the soil, in water, in fæces, in sewage, in dust, and, in fact, practically everywhere, bacteria that under artificial cultivation show no evidence of life at a temperature lower than 60° to 65° C., and will even grow at as high a temperature as 70° to 75° C., degrees of heat sufficient for the coagulation of albumin! Rabinowitsch[4] has likewise described a number of species of these *thermophilic* bacteria, as they are called; but states that it was possible in her experiments to obtain evidence of their growth at a lower temperature (34° to 44° C.), as well as at the higher temperature mentioned by preceding investigators.

The most favorable temperature for the development of pathogenic bacteria is that of the human body, viz.,

[1] **Globig**: Zeitschrift für Hygiene, Bd. iii. S. 294.

[2] **Miquel**: Annales de Micrographie, 1888, pp. 4 to 10.

[3] **Macfadyen and Bloxall**: Journal of Path. and Bact., vol. iii. part i.

[4] **Rabinowitsch**: Zeitschrift für Hygiene u. Infectionskrankheiten, **Bd. xx. Heft 1, S. 154 to 164.**

37.5° C. There are a number of bacteria commonly present in water, the so-called normal *water bacteria*, that grow best at about 20° C.

Under natural conditions it frequently occurs that the development of one species or group of species of bacteria is directly dependent upon the functional activities of another totally distinct species, the growth of one group resulting in conditions that are of vital importance to the existence of the other. This interdependence of species is known as *symbiosis*. It is observed, for instance, in the course of putrefaction, where, through exhaustion of free oxygen by the actively germinating aërobic varieties, the conditions are supplied that enable the anaërobic species to develop and exercise their biological activities. Again, through the proteolytic activity of enzymes produced by certain species of bacteria, other species are supplied with nutrition that would otherwise be unassimilable or only imperfectly so. Similar symbiotic relations between bacteria and higher plants are also noticed, notably that between certain bacteria of the soil and the group of leguminous plants, whereby the latter are enabled, through the assistance of the former, to make up their nitrogen deficit in large part from the free nitrogen of the atmosphere. (See page 35.)

Another interesting biological peculiarity of bacteria is that discovered by Pfeffer, known as *chemotaxis*. This term indicates peculiar phenomena of attraction and of repulsion that are exhibited by motile bacteria when in the presence of solutions of bodies of various chemical composition. Thus, for instance, when a neutral fluid (a drop of water) containing motile bacteria is brought in contact with a weak solution of either pep-

tone, sodium chloride, or dextrin, the bacteria are at once attracted toward the solution; this phenomenon is designated "positive chemotaxis." On the other hand, if brought in contact with an acid, an alkaline, or an alcoholic solution, the bacteria are repelled or driven from the point at which the two fluids are diffusing; this phenomenon is designated "negative chemotaxis." In this connection it is important to note that the wandering cells of the animal body, the leucocytes, exhibit also these chemotactic phenomena; and it is especially necessary to a complete comprehension of the process of suppuration to bear in mind that among the substances which have the greatest attraction for these wandering cells, are the products of growth of certain bacteria in some cases, and the protoplasmic constituents of the bacteria themselves in others.

From what has been learned, it may be said, in general, that for the growth and development of bacteria organic matter of a neutral or slightly alkaline reaction, in the presence of moisture and at a suitable temperature, is necessary. From this can be formed some idea of the omnipresence in nature of these minute vegetable forms. Wherever these conditions obtain bacteria may be found.

CHAPTER II.

Morphology[1] of bacteria—Grouping—Mode of Multiplication—Spore-formation—Motility.

In structure the bacteria are unicellular, always developing from pre-existing cells of the same character and never appearing spontaneously. They are seen to occur as spherical, rod- and spiral-shaped bodies that multiply by the simple process of transverse division, belonging, therefore, to the schizomycetes or *fission fungi.*

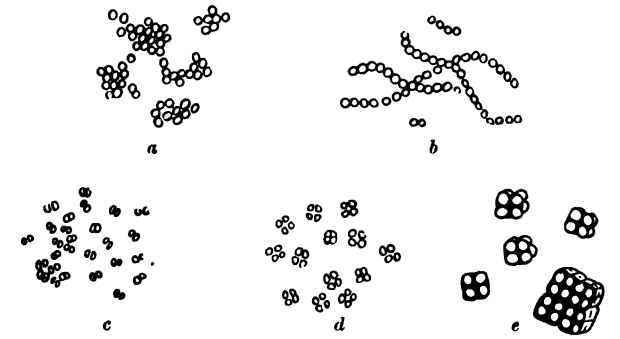
The classifications of the older authors and of the botanists being usually upon purely morphological peculiarities, and, as a rule, taking into consideration all the slight variations that are seen to occur in the size and shape of one and the same species, and of closely allied species, are more or less complicated. The present tendency is to simplify this morphological classification, and to distribute the bacteria into three great groups, with their subdivisions, the members of each group being identified by their individual outline, viz., that of a sphere, a rod, or a spiral. To these three grand divisions are given the names cocci or micrococci, bacilli, and spirilla.

In the group *micrococci* belong all spherical forms—*i. e.*, all those forms the isolated individual members of which are practically of the same diameter in all directions. (See Fig. 1, *a*, *b*, *c*, *d*, *e*.)

[1] Morphology: pertaining to shape, outline, structure.

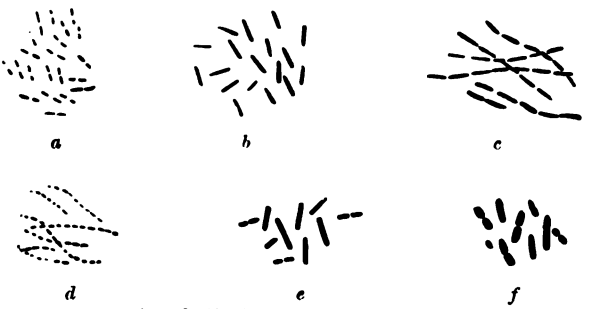
The *bacilli* comprise all oval or rod-formed bacteria. (See Fig. 2.)

FIG. 1.



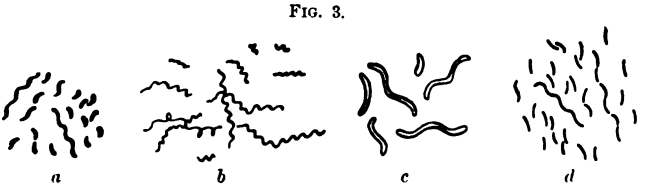
a. Staphylococci. *b*. Streptococci. *c*. Diplococci. *d*. Tetrads. *e*. Sarcinæ.

FIG. 2.



a. Bacilli in pairs. *b*. Single bacilli. *c* and *d*. Bacilli in threads. *e* and *f*. Bacilli of variable morphology.

FIG. 3.



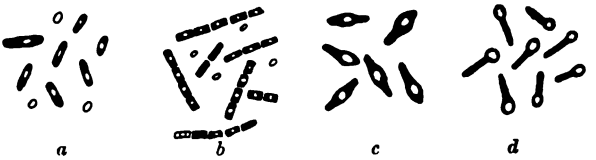
a and *d*. Spirilla in short segments and longer threads—the so-called comma forms and spirals. *b*. The forms known as spirochæta. *c*. The thick spirals sometimes known as vibrios.

To the *spirilla* belong all organisms that are curved when seen in short segments, or when in longer threads are twisted in the form of a corkscrew. (See Fig. 3.)

The micrococci are subdivided according to their prevailing mode of grouping, as seen in growing cultures, into *staphylococci*—those growing in masses like clusters of grapes (see Fig. 1, *a*); *streptococci*—those growing in chains consisting of a number of individuals strung together like beads upon a string (see Fig. 1, *b*); *diplococci*—those growing in pairs (Fig. 1, *c*); *tetrads*—those developing as fours (Fig. 1, *d*); and *sarcinæ*—those dividing into fours, eights, etc., as cubes—that is, in contradistinction to all other forms, the segmentation, which is rarely complete, takes place regularly in three directions of space, so that when growing the bundle of segmenting cells presents somewhat the appearance of a bale of cotton (Fig. 1, *e*).

To the bacilli belong all straight, rod-shaped bacteria —*i. e.*, those in which one diameter is always greater than the other.

FIG. 4.

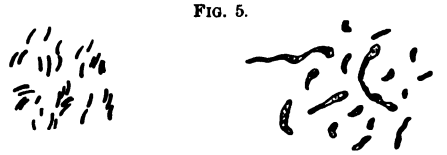


a. Bacillus subtilis with spores. *b*. Bacillus anthracis with spores. *c*. Clostridium form with spores. *d*. Bacillus of tetanus with end spores.

In this group are found those organisms the life-cycle of many of which presents deviations from the simple rod-shape. Many of them in the course of development increase in length into long threads, along which traces of segmentation may usually be

found—the anthrax bacillus and bacillus subtilis are conspicuous examples of this. Again, under certain conditions, many of them possess the property of forming within the body of the rods oval, glistening spores (see Fig. 4), and, if the conditions are not altered, the rods may entirely disappear and nothing be left in the culture but these oval spores. In some of them this phenomenon of spore-formation is accompanied by an enlargement or swelling of the bacillus at the point at which the spore is located (see Fig. 4, *c* and *d*). Again, many of them, from unfavorable conditions of nutrition, aëration, or temperature, undergo pathological changes—that is, the individuals themselves experience degeneration of their protoplasm with coincident distortion of their outline; they are then usually referred to as "involution-forms" (see Fig. 5, *a* and *b*). In

FIG. 5.



a *b*

a. Spirillum of Asiatic cholera (comma bacillus). Involution-forms of this organism as seen in old cultures.

all of these conditions, however, so long as death has not occurred, it is possible to cause these forms to revert to the typical rods from which they originated, by the renewal of the conditions favorable to their normal vegetation.

It must be borne in mind, though, that it is never possible by any means to bring about changes in these organisms that will result in the permanent conversion of the morphology of the members of one group into

that of another—that is, one can never produce bacilli from micrococci, nor *vice versa;* and any evidence which may be presented to the contrary is based upon untrustworthy methods of observation.

Not infrequently bacteria may be observed irregularly massed together as a pellicle. When in this condition they are held together by a gelatinous material, and are known as *zoöglœa of bacteria.* (See Fig. 6.)

FIG. 6.

Zoöglœa of bacilli.

Very short oval bacilli may sometimes be mistaken for micrococci, and at times micrococci in the stage of segmentation into diplococci may be mistaken for short bacilli; but by careful inspection it will always be possible to detect a continuous outline along the sides of the former, and a slight transverse indentation or partition-formation between the segments of the latter. The high index of refraction of spores, the property which gives to them their glistening appearance, will always serve to distinguish them from micrococci. This difference in refraction is especially noticeable if the illumination of the microscope be reduced to the smallest possible bundle of light-rays. The spores, moreover, take up staining-reagents much less readily than do the micrococci. The most reliable differential points, however, are the infallible properties possessed by the spores of developing into bacilli, and by the spherical

organism with which they may have been confounded of always producing other micrococci of the same round form.

A convenient classification of bacilli is that based upon constant characteristics which are seen to appear in the course of their development under special conditions—certain of them possessing the power of forming spores, while from others this peculiarity is absent.

We have less knowledge of the life-history of the spiral forms. Efforts toward their cultivation under artificial conditions have thus far been successful in only a comparatively limited number of cases. Morphologically, they are thread- or rod-like bodies which are twisted into the form of spirals. In some of them the turns of the spiral are long, in others quite short. They are motile and multiply apparently by the simple process of fission.[1] In most respects, save form and the property of producing spores, they are analogous in their mode of growth to the bacilli.

The micrococci multiply by simple fission. When development is in progress a single cell will be seen to elongate slightly in one of its diameters. Over the centre of the long axis thus formed will appear a slight indentation in the outer envelope of the cell; this indentation will increase in extent until there exist eventually two individuals which are distinctly spherical, as was the parent from which they sprang, or they will remain together for a time as diplococci; the surfaces now in juxtaposition are flattened against one another, and not infrequently a fine, pale dividing-line may be seen

[1] Dividing into two transversely.

between the two cells. (See Fig. 1, *c* and *d*.) A similar division in the other direction will now result in the formation of groups of fours as tetrads.

In the formation of staphylococci such division occurs irregularly in all directions, resulting in the production of the clusters in which these organisms are commonly seen. (See Fig. 1, *a*.) With the streptococci, however, the tendency is for the segmentation to continue in one direction only, resulting in the production of long chains of 4, 8, and 12 individuals. (See Fig. 1, *b*.)

The sarcinæ divide more or less regularly in three directions of space; but instead of becoming separated the one from the other as single cells, the tendency is for the segmentation to be incomplete, the cells remaining together in masses. The indentations upon these masses or cubes, which indicate the point of incomplete fission, give to these bundles of cells the appearance commonly ascribed to them—that of a bale of cotton or a packet of rags. (See Fig. 1, *e*.)

The multiplication of bacilli is in the main similar to that given for the micrococci. A dividing cell will elongate slightly in the direction of its long axis; an indentation will appear about midway between its poles, and will become deeper and deeper until eventually two daughter-cells will be formed. This process may occur in such a way that the two young bacilli will adhere together by their adjacent ends in much the same way that sausages are seen to be held together in strings (Fig. 2, *f*), or the segmentation may take place more at right angles to the long axis, so that the proximal ends of the young cells are flattened, while the distal extremities may be rounded or slightly pointed (Fig. [illegible]). The segmentation of the anthrax bacillus, with

which we are to become acquainted later, results, when completed, in an indentation of the adjacent extremities of the young segments, so that by the aid of high magnifying powers these surfaces are seen to be actually concave. Bacilli never divide longitudinally.

With the spore-forming bacilli, under favorable conditions of nutrition and temperature, the same is seen to occur during vegetation; but as soon as these conditions become altered by the exhaustion of nourishment, the presence of detrimental substances, unfavorable temperatures, etc., there appears in their life-cycle the stage to which we have referred as *spore-formation.* This is the process by which the organisms are enabled to enter a state in which they resist deleterious influences to a much higher degree than is possible for them when in the growing or vegetative condition.

In the spore, resting, or permanent state, as it is variously called, no evidence of life whatever is given by the spores; though as soon as the conditions which favor their germination have been renewed these spores develop again into the same kind of cells as those from which they originated, and the appearances observed in the vegetative or growing stage of their history are repeated.

Multiplication of spores, *as such*, does not occur; they possess the power of developing into individual rods of the same nature as those from which they were formed, *but not of giving rise to a direct reproduction of spores.*

When the conditions which favor spore-formation present, the protoplasm of the vegetative cells is seen to undergo a change. It loses its normal homogeneous appearance and becomes marked by granular, refractive points of irregular shape and size. These eventually

coalesce, leaving the remainder of the cell clear and transparent. When this coalescence of highly refractive particles is complete the spore is perfected. In appearance the spore is oval or round, and very highly refractive—glistening. It is easily differentiated from the remainder of the cell, which now consists only of a cell-membrane and a transparent, clear space which surrounds the spore. Eventually both the cell-membrane and its fluid contents disappear, leaving the oval spore free; it then gives the impression of being surrounded by a dark, sharply defined border. It evinces no motion other than the mechanical tremor common to all insoluble microscopic particles suspended in fluids, and it remains quiescent until there appear conditions favorable to its subsequent development into a vegetative form similar to that from which it originated. Occasionally the membrane of the vegetative cell in which the spore is formed does not disappear from around it, and the spore may then be seen lying in a very delicate tubular envelope. Now and then, remnants of the envelope may be noticed adhering to a spore which has not yet become completely free.

By the ordinary methods of staining spores do not become colored, so that they appear in the stained cells as pale, transparent, oval bodies, surrounded by the remainder of the cell, which has taken up the dye.

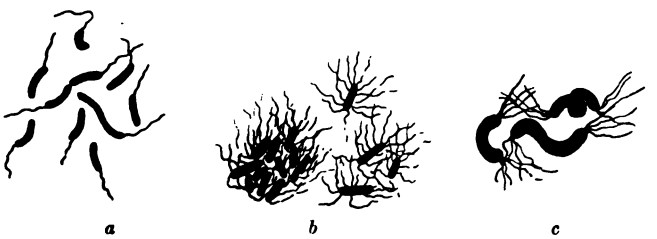
A single cell produces but one spore. This may be located either at an extremity or in the centre of the cell. (Fig. 4.)

Occasionally spore-formation is accompanied by an enlargement of the cell at the point at which the process is in progress. As a result, the outline of the cell loses its regular rod shape and becomes that of a club,

a drum-stick, or a lozenge, depending upon whether the location of the spore is to be at the pole or in the centre of the cell. (See Fig. 4, *e* and *d*.)

In addition to the property of spore-formation there is another striking difference between various species of the rod-shaped organisms, namely, the property of *motility*, by which some of them are distinguished. This power of motion is due to very delicate, hair-

FIG. 7.



a *b* *c*

a. Spiral forms with a flagellum at only one end. *b*. Bacillus of typhoid fever with flagella given off from all sides. *c*. Large spirals from stagnant water with wisps of flagella at their ends (*spirillum undula*).

like appendages or flagella, by the lashing motions of which the rods possessing them are propelled through the fluid. In some cases the flagella are located at but one end of a bacillus, either singly or in a bunch; again, they may be seen at both poles; and in some cases, especially with the bacillus of typhoid fever, they are given off from the whole surface of the rod. (See Fig. 7.) In a few instances similar locomotive organs have been detected on spherical bacteria—*i. e.*, motile micrococci have been observed.

For a long time this property of independent motion was assumed to be due to the possession of some such form of locomotive apparatus, because similar appendages

had been seen upon some of the large, motile spirilla found in stagnant water, but it was not until recently that the accuracy of this assumption was actually demonstrated. By a special method of staining Löffler has been able, in a number of cases, to render visible these hair-like appendages. His method consists in the employment of a mordant, by the aid of which the flagella are caused to retain the staining and thus become visible. Löffler's method of staining will be found in the chapter devoted to this part of the technique.

CHAPTER III.

Principles of sterilization by heat—Methods employed—Discontinued sterilization—Sterilization under pressure—Apparatus employed—Chemical disinfection and sterilization.

Of fundamental importance to successful bacteriological manipulations are acquaintance with the principles underlying the methods of sterilization and disinfection, and familiarity with the approved methods of applying these principles in practice.

In many laboratories it is customary to employ the term sterilization for the destruction of bacteria by heat, and the term disinfection for the accomplishment of the same end through the use of chemical agents. This distinction in the use of the terms is not strictly correct, as we shall endeavor to explain.

The laboratory application of the word sterilization for the destruction of bacteria by high temperatures probably arose from the circumstance that culture-media, and certain other articles that it is desirable to render absolutely free from bacterial life, are not treated by chemical agents for this purpose, but are exposed to the influence of heat in various forms of apparatus known as sterilizers; and the process is, therefore, known as sterilization. On the other hand, cultures no longer useful, bits of infected tissue, and apparatus generally that it is desirable to render free from danger, are commonly subjected for a time to the action of chemical compounds possessing germicidal properties—*i. e.*,

to the action of disinfectants; and the process is, therefore, known as disinfection, though the same end can also be reached by the application of heat to these articles. Strictly speaking, sterilization implies the *complete* destruction of the vitality of all micro-organisms that may be present in or upon the substance to be sterilized, and can be accomplished by the proper application of both thermal and chemical agents; while disinfection, though it may, need not of necessity, insure the destruction of *all* living forms that are present, but only of those possessing the power of *infecting;* it may or may not, therefore, be complete in the sense of sterilization. From this we see it is possible to accomplish both sterilization and disinfection as well by chemical as by thermal means.

In practice the employment of these means is governed by circumstances. In the laboratory it is essential that all culture-media with which work is to be conducted should be free from living bacteria or their spores—they must be sterile; and it is equally important that their original chemical composition should remain unchanged. It is evident, therefore, that sterilization of these substances by means of chemicals is out of the question, for, while the media *could* be thus sterilized, it would be necessary, in order to accomplish this, to add to them substances capable not only of destroying all micro-organisms present, but whose presence would at the same time prevent the growth of bacteria that are to be subsequently cultivated in these media—that is to say, after performing their sterilizing or germicidal function the chemical disinfectants would, by their further presence, exhibit their *antiseptic* properties and thus render the material useless as a culture-

medium. Exceptions to this are seen, however, in the case of certain volatile substances possessing disinfectant powers—chloroform and ether, for instance; these bodies, after exhibiting their germicidal activities, may be driven off by gentle heat, leaving the media quite suitable for purposes of cultivation. They are not, however, in general use in this capacity.

The circumstances under which *chemical* sterilization or disinfection is practised in the laboratory are, ordinarily, either those in which it is desirable to render materials free from danger that are not affected by the chemical action of the agents used, such as glass apparatus, etc., or where destructive changes in the composition of the substances to be treated, as in the case of old cultures, infected tissues, pathological exudates, fæces, etc., are a matter of no consequence. On the other hand, for the sterilization of all materials to be used as culture-media heat only is employed.[1]

The two processes will be explained in this chapter, beginning with

STERILIZATION BY HEAT.

Sterilization by means of high temperature is accomplished in several ways, viz., by subjecting the articles to be treated to a high temperature in a properly constructed oven—this is known as *dry* sterilization; by subjecting them to the action of streaming or live steam at the temperature of 100° C.; and by subjecting them to the action of steam under pressure, under which

[1] An occasional exception to this is the use of chloroform, mentioned above.

circumstance the temperature to which they are exposed becomes more and more elevated as the pressure increases.

Experience has taught us that the process of sterilization by dry heat is of limited application because of its many disadvantages. For successful sterilization by the method of dry heat not only is a relatively high temperature essential, but the substances under treatment must be exposed to this temperature for a comparatively long time. The penetration of dry heat into materials which are to be sterilized is, moreover, much less thorough than that of steam. Many substances of vegetable and animal origin are rendered valueless by subjection to the dry method of sterilization. For these reasons comparatively few materials can be sterilized in this way without seriously impairing their further usefulness.

Successful sterilization by dry heat cannot usually be accomplished at a temperature lower than 150° C., and to this degree of heat the objects should be subjected for not less than one hour. For the sterilization, therefore, of the organic materials of which the media employed in bacteriological work are composed, and of domestic articles, such as cotton, woollen, wooden, and leather articles, this method is wholly unsuitable. In bacteriological work its application is limited to the sterilization of glassware principally—such, for example, as flasks, plates, small dishes, test-tubes, pipettes—and such metal instruments as are not seriously injured by the high temperature.

Sterilization by moist heat—steam—offers conditions much more favorable. The penetrating power of the

steam is not only more complete, but the temperature at which sterilization is ordinarily accomplished is, as a rule, not destructive to the objects under treatment. This is conspicuously seen in the work of the laboratory; the culture-media, composed in the main of decomposable organic materials that would be rendered entirely worthless if exposed to the dry method of sterilization, sustain no injury whatever when intelligently subjected to an equally effective sterilization with steam. The same may be said of cotton and woollen fabrics, bedding, clothing, etc.

Aside from the relations of the two methods to the materials to be sterilized, their action toward the organisms to be destroyed is quite different. The penetrating power of steam renders it by far the more efficient agent of the two. The spores of several organisms which are killed by an exposure of but a few moments to the action of steam, resist the destructive action of dry heat at a higher temperature for a much greater length of time.

These differences will be strikingly brought out in the experimental work on this subject. For our purposes it is necessary to remember that the two methods have the following applications:

The dry method, at a temperature of 150°–180° C. for one hour, is employed for the sterilization of glassware, such as flasks, test-tubes, culture-dishes, pipettes, plates, etc.

Sterilization by steam is practised with all culture-media, whether fluid or solid. Bouillon, milk, gelatin, agar-agar, potato, etc., are under no circumstances to be subjected to dry heat.

The manner in which heat is employed in processes

of sterilization varies with circumstances. When used in the dry form its application is always continuous—*i. e.*, the objects to be sterilized are simply exposed to the proper temperature for the length of time necessary to destroy all living organisms which may be upon them. With the use of steam, on the other hand, the articles to be sterilized are frequently of such a nature that a prolonged application of heat might materially injure them. For this and other reasons steam is usually applied intermittently and for short periods of time. The principles involved in the intermittent method of sterilization depend upon differences of resistance to heat which the organisms to be destroyed are known to possess at different stages of their development. During the life-cycle of many of the bacilli there is a stage in which the resistance of the organism to the action of both chemical and thermal agents is much greater than at other stages of their development. This increased power of resistance appears when these organisms are in the *spore-* or *resting-stage*, to which reference has already been made. When in the vegetative or growing stage most bacteria are killed in a short time by a relatively low temperature; whereas, under conditions which favor the production of spores, the spores are seen to be capable of resisting very much higher temperatures for an appreciably longer time; indeed, spores of certain bacilli have been encountered that retain the power of germinating after an exposure of from five to six hours to the temperature of boiling water. Such powers of resistance have never been observed in the vegetative stage of development. These differences in resistance to heat which the spore-forming organisms possess at their different stages of development are

taken advantage of in the process of sterilization by steam known as the *fractional* or *intermittent method*, and are the essential feature of the principles on which the method is based.

As culture-media are dependent for their usefulness upon the presence of more or less unstable organic compounds, the object aimed at in this method is to destroy the organisms in the shortest time and with the least amount of heat. It is accomplished by subjecting them to the elevated temperature at a time when the bacteria are in the vegetating or growing stage —*i. e.*, the stage at which they are most susceptible to detrimental influences. In order to accomplish this it is necessary that there should exist conditions of temperature, nutrition, and moisture which favor the vegetation of the bacilli and the germination of any spores that may be present. When, as in freshly prepared nutrient media, these surroundings are found, the spore-forming organisms are not only less likely to enter the spore-stage than when their environments are less favorable to their vegetation, but spores which may already exist develop very quickly into mature cells.

It is plain, then, that with the first application of steam to the substance to be sterilized the mature vegetative forms are destroyed; while certain spores that may be present resist this treatment, providing the sterilization is not continued for too long a time. If now the sterilization is discontinued, and the material which presents conditions favorable to the germination of the spores is allowed to stand for a time, usually for about twenty-four hours, at a temperature of from 20° to 30° C., those spores which resisted the action of the steam will, in the course of this interval, germinate

into the less resistant vegetative cells. A second short exposure to the steam kills these forms in turn, and by a repetition of this process all bacteria that were present may be destroyed without the application of the steam having been of long duration at any time. It should be remembered that while spores which may be present are not directly killed by such an exposure to heat as they experience in the intermittent method of sterilization, still their power of germination is somewhat inhibited by this treatment. In this method, therefore, if the temperature of 100° C. be employed for too long a time, it is possible so to retard the germination of the spores as to render it impossible for them to develop into the vegetative stage during the interval between the heatings. By excessively long exposures to high temperature, but not long enough to destroy the spores directly, the object aimed at in the method may be defeated, and in the end the substance undergoing sterilization be found still to contain living bacteria. In this process the plan that has given most satisfactory results is to subject the materials to be sterilized to the action of steam, under the ordinary conditions of atmospheric pressure, for fifteen minutes on each of three successive days, and during the intervals to maintain them at a temperature of about 25°–30° C. At the end of this time all living organisms which were present will, as a general rule, have been destroyed, and, unless opportunity is given for the access of new organisms from without, the substances thus treated remain sterile. As an exception to this, certain species of spore-forming bacteria are occasionally encountered that are not readily destroyed by this mode of treatment. They are, presumably,

of the group of so-called *soil organisms*, and represent the forms most resistant to the influence of heat. We are not as yet sufficiently familiar with all their peculiarities to warrant our speaking with certainty as to a means of sterilizing media in which they are present. It does not seem unlikely that they are of the thermophilic (possibly facultative thermophilic) variety, and therefore show little tendency to develop into the vegetative stage between the heatings, germinating perhaps so slowly at the temperature under which they find themselves as not to leave completely the spore-stage before another exposure to the steam, but manifesting after a time properties of life in media that are thought to be sterile and which have been placed aside for subsequent use. This is a mere hypothesis, however, and is as yet entirely wanting in experimental proof.

Theobald Smith [1] has recently called attention to an instructive personal experience. He finds that when media are present in vessels in only thin layers the spores of *anaërobic* species do not develop into the vegetative forms during the interval between the heatings, for the reason that the shallow layer of medium does not sufficiently exclude free oxygen to permit it; and by subjecting such materials, *apparently* sterilized by the intermittent method, to strictly anaërobic conditions a development of anaërobic species will often occur. On the other hand, if the vessels be nearly filled with media, and especially if the area of the surface be small, the conditions are much more favorable to the germination of anaërobic spores and sterilization by this process is usually perfect.

[1] Theobald Smith: Journal of Experimental Medicine, vol. iii. No. 6, p. 647.

Fortunately, these undesirable experiences are rare, but that they do occur, and result in no small degree of annoyance, will be admitted by most bacteriologists.

It must be borne in mind that this method of sterilization is only applicable in those cases which present conditions favorable to the germination of the spores into mature vegetative cells. Dry substances, such as instruments, bandages, apparatus, etc., or organic materials in which decomposition is far advanced, where conditions of nutrition favorable to the germination of spores are not present, do not offer the conditions requisite for the successful operation of the principles underlying the intermittent method of sterilization.

The process of fractional sterilization at *low* temperatures is based upon exactly the same principle, but differs in two respects from the foregoing in the manner by which it is practised, viz., it requires a greater number of exposures for its accomplishment, and the temperature at which it is conducted is not above 68°–70° C. It is employed for the sterilization of easily decomposable materials, which would be rendered useless by steam, but which are unaltered by the temperature employed, and for certain albuminous culture-media that it is desirable to retain in a fluid condition during sterilization, but which would be coagulated if exposed to higher temperatures. This process requires that the material to be sterilized should be subjected to a temperature of 68°–70° C. for one hour on each of six successive days, the interval of twenty-four hours between the exposures admitting of the germination of spores into mature cells. During this interval the substances under treatment are kept at about 25°–

30° C. The temperature employed in this process suffices to destroy, in about one hour, the vitality of almost all organisms in the vegetative stage. Formerly blood-serum was always sterilized by the intermittent method at a low temperature.

Sterilization by steam is also practised by what may be called the direct method—that is to say, both the mature organisms and the spores which may be present in the material to be sterilized are destroyed by a single exposure to the steam. In this method steam at its ordinary temperature and pressure—live steam or streaming steam, as it is called—is employed just as in the first method described; but it is allowed to act for a much longer time, usually for not less than an hour; or steam under pressure, and consequently of a higher temperature, is now frequently employed. By the latter procedure a single exposure of fifteen minutes is sufficient for the destruction of practically all bacilli and their spores, providing the pressure of the steam is not less than one atmosphere over and above that of normal; this is approximately equivalent to a temperature of 122° C. to which the organisms are exposed.

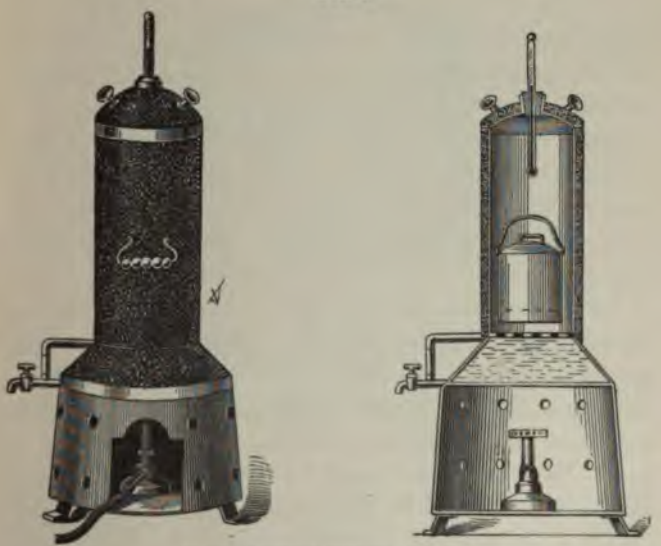
The objection that has been urged to both of these methods, particularly that in which steam under pressure is employed, is that the properties of the media are altered. Gelatin is said to become cloudy and lose the property of solidifying; in bouillon and agar-agar fine precipitates are said to result, and some believe the reaction undergoes a change. In the experience of those who have used steam under pressure not exceeding one or one and one-half atmospheres for ten to fifteen minutes these obstacles have rarely been

encountered. There is one point to be borne in mind, however, in using steam under pressure, viz., it is not possible to regulate the *time of exposure* to the same degree of nicety as where ordinary live steam is used. The reason for this is that if the apparatus be opened to remove the objects being sterilized while the steam within it is under pressure, the escape of steam will be so rapid that all fluids within the chamber, thus suddenly relieved of pressure, will begin to boil violently, and, as a rule, will boil quite out of the tubes, flasks, etc., containing them. For this reason the apparatus must be kept closed until cool, or until the gauge indicates that pressure no longer exists within the chamber, and even then it should be opened very cautiously. It is patent from this that the temperature and time of exposure of articles sterilized by this process cannot usually be controlled with accuracy. It requires some time to reach a given pressure after the apparatus is closed, and it also requires time for cooling after the desired exposure to such pressure before the apparatus can be opened.

It is manifest that during these three periods, viz., (*a*) reaching the pressure desired, (*b*) time during which the pressure is maintained, and (*c*) time for fall of pressure before the chamber can be opened, it is difficult to say certainly to what temperature and pressure the articles in the apparatus have, *on the whole*, been subjected. Clearly, if the desired pressure and temperature have been maintained for ten minutes, one cannot say that this is *all* the heat to which the articles have been subjected during their stay in the chamber. In this light, while steam under pressure may answer very well for routine sterilization, still it presents insurmountable

obstacles to its use in more delicate experiments where time-exposure to definite temperature is of importance. Nevertheless, for general laboratory purposes, sterilization by steam under pressure has so much to recommend it in the way of economy of time and certainty of accomplishment that it has practically superseded the older methods of sterilization by steaming or live

FIG. 8.



Steam sterilizer, pattern of Koch.

steam; and in most laboratories the original styles of steam sterilizers are rapidly giving way to some one or another of the modern forms of autoclave.

For sterilization by live steam the apparatus commonly employed has, until recently, been the cylindrical boiler recommended by Koch. (See Fig. 8.)

Its construction is very simple, essentially that of the ordinary domestic potato-steamer. It consists of a copper cylinder, the lower fifth of which is somewhat larger in circumference than the remaining four-fifths and acts as a reservoir for the water from which the steam is to be generated. Covering this section of the cylinder is a wire rack or grating through which the steam passes, and which serves to support the articles to be sterilized. Above this, comprising the remaining four-fifths of the cylinder, is the chamber for the reception of the materials over and through which the steam is to pass. The cylinder is closed by a snugly fitting cover through which are usually two perforations into which a thermometer and a manometer may be inserted. The whole of the outer surface of the apparatus is encased in a non-conducting mantle of asbestos or felt.

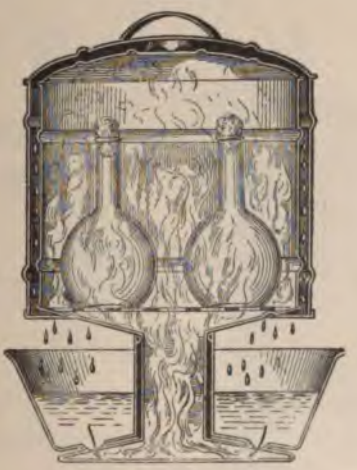
The water is heated by a gas-flame placed in an enclosed chamber, upon which the apparatus rests, which serves to diminish the loss of heat and deflection of the flame through the action of draughts. The apparatus is simple in construction, and the only point which is to be observed while using it is the level of the water in the reservoir. On the reservoir is a water-gauge which indicates at all times the amount of water in the apparatus. The amount of water should never be too small to be indicated by the gauge; otherwise there is danger of the reservoir becoming dry and the bottom of the apparatus being destroyed by the direct action of the flame.

A sterilizer that has come into very general use in bacteriological laboratories is one originally intended for use in the kitchen. It is called the "Arnold steam

sterilizer." It is very ingenious in its construction as well as economical in its employment.

The difference between this apparatus and that just described is that it provides for the condensation of the steam after its escape from the sterilizing chamber, and returns the water of condensation automatically to the

FIG. 9.



Arnold steam sterilizer.

reservoir, so that in practice the apparatus requires but little attention, as with ordinary care there is no likelihood of the water in the reservoir becoming exhausted, with the consequent destruction of the sterilizer.

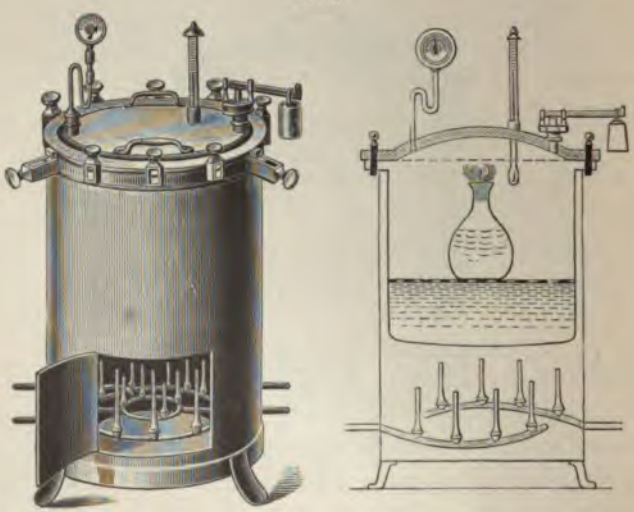
Fig. 9 shows a section through this apparatus.

STERILIZATION UNDER PRESSURE.

The advantages of the use of steam under pressure for the purposes of sterilization have received such general recognition that almost everywhere this method

is supplanting the older one of intermittent sterilization with steaming or live steam. By this plan one is able to accomplish, by a single exposure of fifteen minutes to steam under a pressure of one atmosphere, the same end that would, with steaming steam, require three exposures of fifteen minutes on each of three successive days.

FIG. 10.



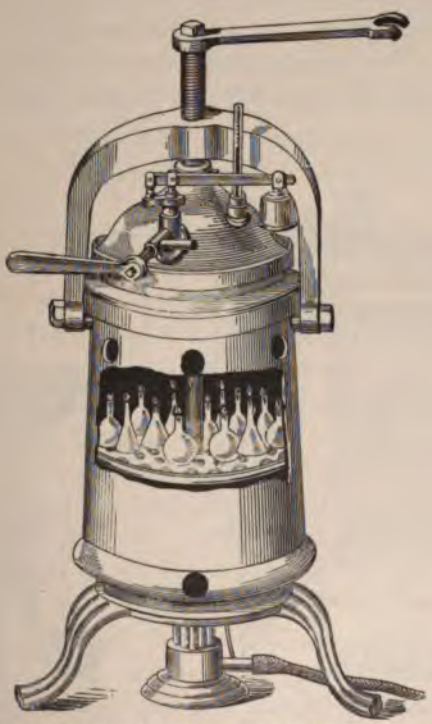
A *B*

Autoclave, pattern of Wiesnegg. *A*. External appearance. *B*. Section.

For sterilization by steam *under pressure* several special forms of apparatus exist. The principles involved in them all are, however, the same. They provide for the generation of steam in a chamber from which it cannot escape when the apparatus is closed. Upon the cover of this chamber is a safety-valve, which can be regulated so that any degree of pressure (and coincidently of temperature) that is desirable may be main-

tained within the sterilizing chamber. These sterilizers are known as "digesters" and as "autoclaves." Their

FIG. 11.



Autoclave or digester for sterilizing by steam under pressure.

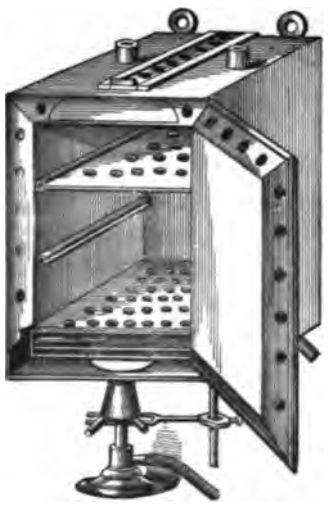
construction can best be understood by reference to Figs. 10 and 11.

STERILIZATION BY HOT AIR.

The hot-air sterilizers used in laboratories are simply double-walled boxes of Russian or Swedish iron

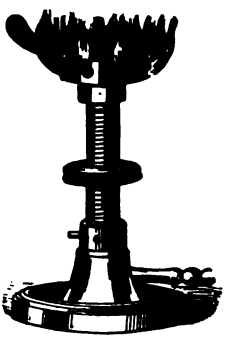
(Fig. 12), having a double-walled door, which closes tightly, and a heavy copper bottom. They are provided with openings for the escape of the contained air and the entrance of the heated air. The flame, usually from a rose-burner (Fig. 13), is applied directly to the bottom. The heat circulates from the lower surface around about the apparatus through the space between its walls.

FIG. 12.



Laboratory hot-air sterilizer.

FIG. 13.



Rose-burner.

The construction of the copper bottom of the apparatus upon which the flame impinges is designed to prevent the direct action of the flame upon the sheet-iron bottom of the chamber. It consists of several copper plates placed one above the other, but with a space of about 4 to 5 mm. between the plates. These copper bottoms after a time become burned out, and unless

they are replaced the apparatus is useless. The older forms of hot-air sterilizers are so constructed that their repair is a matter involving some time and expense. To meet this objection I have had constructed a sterilizer in all respects similar to the old form except in the arrangement of the copper bottom. This is made in such a way that it can be easily removed, so that by keeping several sets of copper plates on hand a new plate can readily be inserted when the old one is burned out.

In the employment of the hot-air sterilizer care should always be given to the condition of the copper bottom; for the direct application of heat to the sheet-iron plate upon which the substances to be sterilized stand results not only in destruction of the apparatus, but frequently in destruction of the substances undergoing sterilization.

Since the temperature at which this form of sterilization is usually accomplished is high, from 150° to 180° C., it is well to have the apparatus encased in asbestos boards, to diminish the radiation of heat from its surfaces. This not only confines the heat to the apparatus, but guards against the destructive action of the radiated heat on woodwork, furniture, etc., that may be in the neighborhood.

CHEMICAL STERILIZATION AND DISINFECTION.

As has been stated, it is possible by means of certain chemical substances to destroy all bacteria and their spores that may be within or upon various materials and objects—*i. e.*, to sterilize them; and it is also possible by the same means to rob objects of their

dangerous infective properties without at the same time sterilizing them—*i. e.*, to disinfect them. This latter process depends upon the fact that the vitality of many of the less resistant *pathogenic* organisms is easily destroyed by an exposure to particular chemical substances that may be without effect upon the more resistant saprophytes and their spores that are present.

In general, the use of chemicals for sterilization is not to be considered in connection with substances that are to be employed as culture-media, and their employment is restricted in the laboratory to materials that are of no further value, and to infected articles that are not injured by the action of the agents used, though exceptionally such volatile germicides as chloroform and ether are employed for the sterilization of special culture-media. (See Preservation of Blood-serum with Chloroform.) In short, they are mainly of value in rendering infected waste-materials innocuous. For the successful performance of this form of disinfection there is one fundamental rule always to be borne in mind, viz., it is absolutely essential to success that the disinfectant used should come in direct contact with the bacteria to be destroyed, otherwise there is no disinfection.

For this reason, one should always remember, in selecting the disinfecting agent, the nature of the materials containing the bacteria upon which it is to act, for the majority of disinfectants, and particularly those of an inorganic nature, vary in the degree of their potency with the chemical nature of the mass to which they are applied. Often the materials containing the bacteria to be destroyed are of such a character that they combine with the disinfecting agent to form insoluble pre-

cipitates; these so interfere with the penetration of the disinfectant that many bacteria may escape its destructive action entirely and no disinfection be accomplished, though an agent may have been employed that would, under other circumstances, have given entirely satisfactory results.

In the destruction of bacteria by means of chemical substances there occurs, most probably, a definite chemical reaction—that is to say, the characteristics of both the bacteria and the agent employed in their destruction are lost in the production of an inert third body, the result of their combination. It is impossible to say with absolute certainty, as yet, that this is the case; but the evidence that is rapidly accruing from the more recent studies upon disinfectants and their mode of action points strongly to the accuracy of this belief. This reaction, in which the typical structures of both bodies concerned are lost, takes place between the agent employed for disinfection and the protoplasm of the bacteria. For example, in the reaction that is seen to take place between the salts of mercury and albuminous bodies there results a third compound, which has neither all the characteristics of mercury nor of albumin, but partakes of the peculiarities of both; it is a combination of albumin and mercury, commonly known by the indefinite term "albuminate of mercury." Some such reaction as this apparently occurs when the soluble salts of mercury are brought in contact with bacteria. This view has been strengthened by the experiments of Geppert, in which the reaction was caused to take place between the spores of the anthrax bacillus and a solution of mercuric chloride, the result being the *apparent* destruction of the vitality of the spores by the forma-

tion of this third compound. In these experiments it was shown that though this combination had taken place, still it did not of necessity imply the death of the spores, for if by proper means the combination of mercury with their protoplasm was broken up, many of the spores resumed their vitality, with all their previous disease-producing and cultural peculiarities. Geppert employed a solution of ammonium sulphide for the purpose of destroying the combination of spore-protoplasm and mercury; the mercury was precipitated from the protoplasm as an insoluble sulphide, and the protoplasm of the spores returned to its original condition. These and other somewhat similar experiments have given a new impulse to the study of disinfectants, and in the light shed by them many of our previously formed ideas concerning the action of disinfecting agents must be modified.

The process of disinfection is not a catalytic one—*i. e.*, occurring simply as a result of the presence of the disinfecting body, which is not itself decomposed during its process of destruction—but is, as said, a definite chemical reaction occurring within more or less fixed limits; that is to say, with a given amount of the disinfectant employed so much work, expressed in terms of disinfection—destruction of bacteria—can be accomplished.

Another point in favor of this view is the increased energy of the reaction with elevation of temperature. Just as in many other chemical phenomena the intensity and rapidity of the reaction become greater under the influence of heat, so in the process of disinfection the combination between the disinfectant and the organisms to be destroyed is much more energetic at a temperature of 37° to 39° C. than it is at 12° to 15° C.

A number of important and novel suggestions with regard to the *modus operandi* of disinfection have recently been brought out through the work of Krönig and Paul,[1] who took up the subject from its physical-chemical standpoint. The comprehensive nature of this elaborate investigation precludes more than a brief mention of some of the conclusions reached; and in order that these may be intelligible certain beliefs (working hypotheses) of the physical-chemists should be borne in mind. In 1887 Arrhenius proposed the theory that when an electrolyte (a compound decomposable by an electric current) is dissolved in water its molecules break down, not simply into their component atoms, but into *ions*, which are atoms or groups of atoms having electro-positive and electro-negative characteristics. According to this theory, salts, when dissolved in water, undergo electrolytic dissociation into metallic and acidic *ions*, the former being the electro-positive *cation*, the latter the electro-negative *anion;* sodium chloride, for example, resolving itself, under these conditions, into its sodium, or *metal-ion*, and its chlorine, or *acidic ion*. The electro-positive cations, according to Ostwald, comprise the metals and metal-like radicals, such as ammonium (NH_4) and hydrogen (H); while the electro-negative anions include the halogens, the acidic radicals (such as NO_3 and SO_4), and hydroxyl.[2] Using this theory as the basis of their investigations, Krönig and Paul

[1] Krönig and Paul: Zeitschrift für Hygiene und Infectionskrankheiten, 1897, Band xxv. S. 1-112.

[2] Consult Ostwald's Lehrbuch der Allg. Chemie: or Muir's translation of Ostwald's Solutions, p. 189, published by Longmans, Green & Co., London and New York, 1891. Also "The Rise of the Theory of Electrolytic Dissociation," etc., by H. C. Jones, Ph. D., Johns Hopkins Hospital Bulletin, No. 87, June, 1898, p. 136.

reached the following conclusions with regard to the action of chemical disinfectants:

The germicidal value of a metallic salt depends not only upon its specific character, but also upon that of its *anion*.

Solutions of metallic salts in which the metallic part is represented by a complex ion and in which the concentration of the metal ion is very slight, have but feeble disinfecting activity.

The halogen compounds of mercury act according to the degree of their dissociation.

The disinfecting power of the halogens—chlorine, bromine, iodine—(as well as their compounds) is in inverse ratio to their atomic weights.

The disinfecting activity of watery solutions of mercuric chloride is diminished by the addition to them of other halogen compounds of metals and of hydrochloric acid. It appears probable that this is due to obstruction offered to electrolytic dissociation.

The disinfecting activities of watery solutions of mercuric nitrate, mercuric sulphate, and mercuric acetate are increased by the moderate addition of sodium chloride.

In general, acids disinfect according to the degree of their dissociation—*i. e.*, according to the concentration of their hydrogen ions in the solution.

The bases, potassium, sodium, lithium, and ammonium hydroxide, disinfect according to the degree of their dissociation—*i. e.*, corresponding to the concentration of their hydroxyl ions in the solution.

The disinfecting activity of metallic salts is, in general, less in albuminous fluids than in water. It is probable that this is due to a diminution in the concentration of metallic ions in the solution.

What has been said refers more particularly to the inorganic salts which are employed for this purpose. It is probable that the organic bodies possessing disinfectant properties owe this power to some such similar reaction, though, as yet, these substances have not been so thoroughly studied in this relation.

The reaction between the inorganic salts and albuminous bodies is not selective; they combine in most instances with any or all protoplasmic bodies present. For this reason the employment of many of the commoner disinfectants in general practice is a matter of doubtful advantage. For example, the disinfection of excreta, sputum, or blood, containing pathogenic organisms, by means of corrosive sublimate, is a procedure of questionable success. The amount of sublimate employed may be entirely used up and rendered inactive as a disinfectant by the ordinary protoplasmic substances present, without having any appreciable effect upon the bacteria which may be in the mass.

These remarks are introduced in order to guard against the implicit confidence so often placed in the disinfecting value of corrosive sublimate. In many bacteriological laboratories it is the custom to keep at hand vessels containing solutions of corrosive sublimate, into which infectious materials may be placed. The value of this procedure, as we have just learned, may be more or less questionable, especially in those cases in which the substance to be disinfected is of a proteid nature and where the solution used is not freshly prepared. On the introduction of such substances into the sublimate solution the mercury is quickly precipitated by the albumin, and its disinfecting properties may be in large part or entirely destroyed; we may in

a very short time have little else than water containing a precipitate of albumin and mercury, in so far as its value as a disinfectant is concerned.

Though the other inorganic salts have not been so thoroughly studied in this connection, it is nevertheless probable that the same precautions should be taken in their employment as we now know to be necessary in the use of the salts of mercury.

Where it is desirable to use chemical disinfectants in the laboratory much more satisfactory results can usually be obtained from the employment of carbolic acid in solution. A 3 or 4 per cent. solution of commercial carbolic acid in water requires longer for disinfection; but it is, at the same time, open to fewer objections than are solutions of the inorganic salts; though here, too, we find a somewhat analogous reaction between the carbolic acid and proteid matters. Under ordinary circumstances its action is complete in from twenty minutes to one-half hour. It is not reliable for the disinfection of resistant spores; such, for instance, as those of *bacillus anthracis*.

All tissues containing infectious organisms should be *burned*, and all cloths, test-tubes, flasks, and dishes should be boiled in 2 per cent. soda (ordinary washing-soda) solution for fifteen to twenty minutes, or placed in the steam sterilizer for half an hour.

Intestinal evacuations may best be disinfected with boiling water or with *milk of lime*, a mixture composed of lime in solution and in suspension—ordinary fluid "white-wash." This should be thoroughly mixed with the evacuations until the mass reacts distinctly alkaline, and should remain in contact with them for one or two hours. Excreta may also be easily disinfected by

thoroughly mixing them with two or three times their volume of *boiling* water, after which they are kept covered until cool.

Sputum in which tubercle bacilli are present, as well as the vessel containing it, must be boiled in 2 per cent. soda solution for fifteen minutes, or steamed in the sterilizer for at least half an hour.

On the whole, in the laboratory we should as yet rely more upon the destructive properties of heat than upon those of chemical agents.

From what has been said, the absurdity of sprinkling here and there a little carbolic acid, or in placing vessels of carbolic acid about apartments in which infectious diseases are in progress, must be plain. Treatment of water-closets and cesspools by allowing now and then a few cubic centimetres of some so-called disinfectant to trickle through the pipes is ridiculous. A disinfectant must be *applied to the bacteria, and must be in contact with them for a long enough time to insure the destruction of their life.*

In the light of the latest experiments upon disinfectants, the place formerly occupied by many agents in the list of substances employed for the purpose will most likely be changed as they are studied more closely. The agents, then, which will prove of greatest value in the laboratory for the purpose of rendering infectious materials harmless are: *heat*, either by burning, by steaming for from half an hour to an hour, or by boiling in a 2 per cent. sodium carbonate solution for fifteen minutes; 3 to 4 per cent. solution of commercial carbolic acid; *milk of lime*, and a solution of *chlorinated lime* ("chloride of lime") containing not less than 0.25 per cent. of free chlorine. The chloride of lime from

which such a solution is to be made should be fresh and of good quality. Good chlorinated lime, as purchased in the shops, should contain not less than 25 to 30 per cent. of available chlorine. The materials to be disinfected in either of the lime solutions should remain in them for about two hours. The solutions should be freshly prepared when needed, as they rapidly decompose upon standing.

An *antiseptic* is a body which, by its presence, prevents the growth of bacteria without of necessity killing them. A body may be an antiseptic without possessing disinfecting properties to any very high degree, but a disinfectant is always an antiseptic as well.

A *germicide* is a body possessing the property of killing bacteria.

CHAPTER IV.

Principles involved in the methods of isolation of bacteria in pure culture by the plate method of Koch—Materials employed.

As was stated in the introductory chapter, the isolation in pure cultures of the different species that may be present in mixtures of bacteria was rendered possible only through the methods suggested by Koch. Since the adoption of these methods they have undergone many modifications, but the fundamental principle remains the same. The observation which led to their development was a very simple one, and one that is frequently before us. Koch noticed that on solid substances, such, for example, as a slice of potato or of bread, which had been exposed for a time to the air and which afforded proper nourishment for the lower organisms, there developed after a short time small patches which proved to be colonies of bacteria. Each of these colonies on closer examination proved to be, as a rule, composed of distinct species of micro-organisms. There was little tendency on the part of these colonies to become confluent, and from the differences in their naked-eye appearances it was easy to see that they represented, in the main, the development of different species of bacteria.

The question that then presented itself was: If from a mixture of organisms floating in the air it is possible in this way to obtain in pure cultures the component individuals, what means can be employed to obtain the

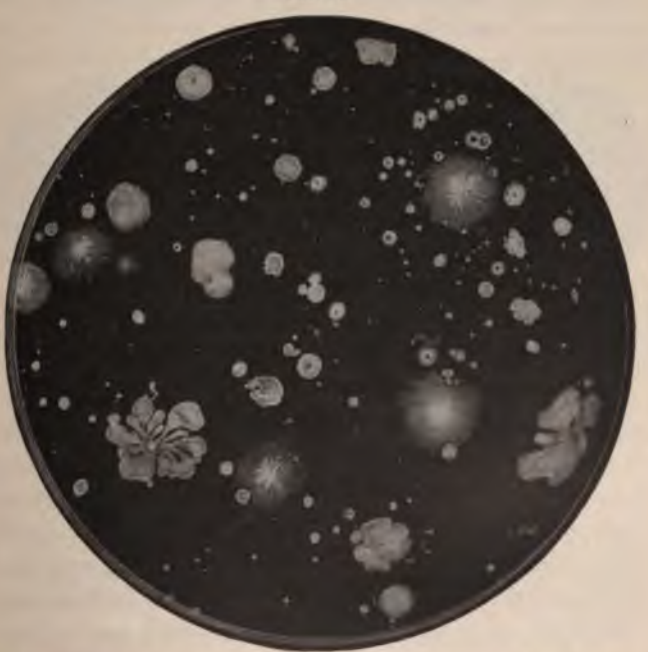
same results *at will* from mixture of different species of bacteria when found together under other conditions? It was plain that the organisms were to be distinguished primarily, the one from the other, only by the structure and general appearance of the colonies growing from them, for by their morphology alone this is impossible. What means might be devised, then, for separating the individual members of a mixture in such a way that they would remain in a fixed position, and be so widely separated, the one from the other, as not to interfere with the production of colonies of characteristic appearance, which would, under favorable conditions, develop from each individual cell?

If one take in the hand a mixture of barley, rye, corn, oats, etc., and attempt to separate the mass into its constituents by picking out the different grains, much difficulty is experienced; but if the handful of grain be thrown upon a large, flat surface, as upon a table, the grains become widely separated and the task is considerably simplified; or, if sown upon proper soil, the various grains will develop into growths of entirely different external appearance, by which they can readily be recognized as unlike in nature. Similarly, if a test-tube of decomposed bouillon be poured upon a large, flat surface, the individual bacteria in the mass are much more widely separated, the one from the other, than they were when the bouillon was in the tube; but they are in a fluid medium, and there is no possibility of their either remaining separated or of their forming colonies under these conditions, so that it is impossible by this means to pick out the individuals from the mixture.

If, however, some substance could be found which

possesses the property of being at one time fluid and at another time solid, and which could be added to this bouillon without in any way interfering with the life-functions of the bacteria, then, as solidification set in, the organisms could be fixed in their positions, and the conditions would be analogous to those seen on the bit of potato.

FIG. 14.



Showing certain macroscopic characteristics of colonies. Natural size.

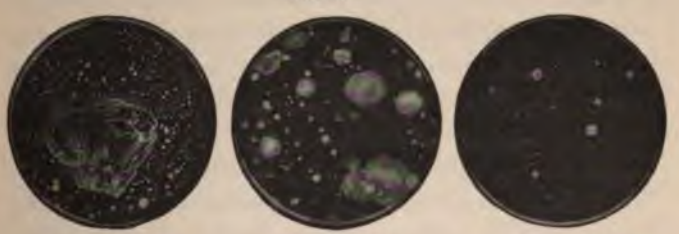
Gelatin possesses this property, and it was, therefore, used. At a temperature which does not interfere with the life of the organisms it is quite fluid, whereas when subjected to a lower temperature it solidifies. When

once solid it may be kept at a temperature favorable to the growth of the bacteria and will remain in its solid condition.

Gelatin was added to the fluids containing mixtures of bacteria, and the whole was then poured upon a large, flat surface, allowed to solidify, and the results noted. It was found that the conditions seen on the slice of potato could be reproduced; that the individuals in the mixture of bacteria grew well in the gelatin, and, as on the potato, grew in colonies of typical macroscopic structures, so that they could easily be distinguished the one from the other by their naked-eye appearances. (See Fig. 14.) It was necessary, however, to use a more dilute mixture of bacteria than the original decomposed bouillon. The number of individuals in the tube was so enormous that on the gelatin plate they were so closely packed together that it was impossible to pick them out, not only because of their proximity the one to the other, but also because this packing together materially interfered with the production of those characteristic differences visible to the naked eye. The numbers of the organisms were then diminished by a process of dilution, consisting of transferring a small portion of the original mixture into a second tube of sterilized bouillon to which gelatin had been added and liquefied; from this a portion was added to a third gelatin-bouillon tube, and so on. These were then poured upon large surfaces and allowed to solidify. The result was entirely satisfactory. On the gelatin plates from the original tube, as was expected, the colonies were too numerous to be of use; on the plates made from the first dilution they were much fewer in number, but usually they were still too

numerous and too closely packed to permit of characteristic growth; on the second dilution they were, as a rule, fewer in number and widely separated, so that the individuals of each species were in no way prevented by the proximity of their neighbors from growing each in its typical way. (See Fig. 15.) There was then no difficulty in picking out the colonies resulting from the growth of the different individual bacteria.

FIG. 15.



A *B* *C*

Series of plates showing the results of dilution upon the number of colonies: *A*. Plate No. 1, or "original." *B*. First dilution, or Plate No. 2. *C*. Second dilution, or Plate No. 3. About one-fourth natural size.

This, then, is the principle underlying Koch's method for the isolation of bacteria by means of solid media.

The fundamental constituent of the media employed is the bouillon, which contains all the elements necessary for the nutrition of most bacteria, the gelatin being employed simply for the purpose of rendering the bouillon solid. The medium on which the organisms are growing is, therefore, simply solidified bouillon, or beef-tea.

In practice, two forms of gelatin are employed—the one an animal or bone gelatin, the ordinary table gelatin of good quality; the other a vegetable gelatin, known as agar-agar, the native name for Ceylon moss or Ben-

gal isinglass, which is obtained from a group of algæ growing in the sea along the coast of Japan, China, and many parts of the East, where it is employed as an article of diet by the natives.

Aside from these differences in origin of the two forms of gelatin employed, their behavior under the influence of heat and of bacterial growth renders them of different application in bacteriological work. The animal gelatin liquefies at a much lower temperature, and also requires a lower temperature for its solidification, than does the agar-agar. Ordinary gelatin, in the proportion commonly used in this work, liquefies at about 24°–26° C., and becomes solid at from 8° to 10° C. It may be employed for those organisms which do not require a higher temperature for their development than 22°–24° C. Agar-agar, on the other hand, does not liquefy until the temperature has reached about 98°–99° C. It remains fluid ordinarily until the temperature has fallen to 38°–39° C., when it rapidly solidifies. For our purposes, only that form of agar-agar can be used which remains fluid at from 38° to 40° C. Agar-agar which remains fluid only at a temperature above this point would be too hot, when in a fluid state, for use; many of the organisms introduced into it would either be destroyed or checked in their development by so high a temperature. Agar-agar is employed in those cases in which the cultivation must be conducted at a temperature above the melting-point of gelatin.

In addition to their differences when under the influence of various temperatures, the relations of these two gelatins to bacteria are quite distinct. Many bacteria bring about alterations in gelatin which cause it to

e liquid (a process analogous to peptonization), in

which state it remains. There are no known organisms that bring about such a change in agar-agar.

As a rule, the colony-formations seen upon gelatin are much more characteristic than those which develop on agar-agar, and for this reason gelatin is to be preferred when circumstances will permit. Both gelatin and agar-agar may be used in the preparation of plates and Esmarch tubes, subsequently to be described.

CHAPTER V.

Preparation of media—Bouillon, gelatin, agar-agar, potato, blood-serum, etc.

As has been stated, the fundamental constituent of culture-media is beef-tea, or bouillon.

BOUILLON.—The directions of Koch for the preparation of this medium have undergone many modifications to meet special cases; but for general use his original formula is still retained. It is as follows: 500 grammes of finely chopped lean beef, free from fat and tendons, are to be soaked in one litre of water for twenty-four hours, during which time the mixture is to remain in an ice-chest or to be otherwise kept at a low temperature. At the end of twenty-four hours it is to be strained through a coarse towel and pressed until a litre of fluid is obtained. To this are to be added 10 grammes (1.0 per cent.) of dried peptone and 5 grammes (0.5 per cent.) of common salt (NaCl). It is then to be rendered exactly neutral or very slightly alkaline with a few drops of saturated sodium carbonate solution. The flask containing the mixture is then to be placed either in a steam sterilizer or in a water-bath, or over a free flame, and kept at the boiling-point until all the albumin is coagulated and the fluid portion is of a clear, pale straw color. It is then filtered through a folded paper filter and sterilized in the steam sterilizer by the fractional method. Certain modifications of this method are of sufficient value to justify mention. Most im-

portant is the neutralization. Ordinarily, this is accomplished with the saturated sodium carbonate solution, and the reaction is determined with red and blue litmus papers; for the beginner this method serves most purposes.

The sodium carbonate solution is not so good, however, as a strong solution of caustic soda or potash, because the carbonic acid liberated from the sodium carbonate frequently gives rise to a confusing, temporary acid reaction which disappears on heating; nor is litmus the most reliable indicator to employ. To obviate this, Schultz (*Centralb. f. Bakt. u. Parasitenkunde*, 1891, Bd. x., Nos. 2 and 3) recommends exact titration with a solution of caustic soda. For this purpose a 4 per cent. solution of caustic soda is prepared. From this a 0.4 per cent. solution is made, and with it the titration is practised. After the bouillon has been deprived of all coagulable albumin and blood-coloring-matter by boiling and filtration, and has cooled down to the temperature of the air, its volume is exactly measured.

From this a sample of exactly 5 or 10 c.c. is then taken, and to it a few drops of one of the indicators commonly employed in analytical work are added. Schultz recommends 1 drop of phenolphtalein solution (1 gramme of phenolphtalein in 300 c.c. of alcohol) to 1 c.c. of bouillon. The beaker containing the sample is placed upon white paper, and the dilute caustic soda solution is then allowed to drop very slowly into it from a burette, until there appears a very delicate rose color, which indicates the beginning of alkaline reaction. A second sample of the bouillon is treated in the same way. If the amounts of caustic soda solution required

for each sample deviate but very slightly or not at all the one from the other, the mean of these amounts is taken as the amount of alkali necessary to neutralize the quantity of bouillon employed. If 10 c.c. of bouillon were employed, then for the whole amount of 1 litre just 100 times as much, minus that for the two samples used in titration, will be needed. For example: to neutralize 10 c.c. of bouillon 2 c.c. of the diluted (0.4 per cent.) caustic soda solution were employed. For the remaining 980 c.c. of the litre of bouillon, then, 196 c.c. (200 c.c. less 4 c.c., the amount employed for the two samples of 10 c.c. each of bouillon) are needed of the 0.4 per cent. solution, or one-tenth of this amount of the 4 per cent. caustic soda solution.

For the neutralization of the whole bulk of the bouillon it is better to employ the stronger alkaline solution, as by its use the volume is not increased to so great an extent as when the dilute solution is used.

It is evident that this method is much more exact than that ordinarily employed; but at the same time it must be remembered that for its success exactness in the measurement of the volumes and in the preparation of the dilutions is required. To obviate error, it is better to employ this method when the solutions are all cool and of nearly the same temperature, so that rapid fluctuations in temperature, and consequent alterations in volume, will not materially interfere with the accuracy of the results.

This method of neutralization, as suggested by Schultz, should be adopted for experiments in which it is desirable to have the reaction of the medium accurate and constantly of the same degree.

For the purposes of the beginner, however, results quite satisfactory in their nature may be obtained by the employment of the saturated sodium carbonate solution for neutralization, with litmus paper as the indicator. For some time, however, it has been our practice to employ the yellow curcuma paper for the detection of alkalinity, rather than the red litmus paper.

In the exhaustive paper of Fuller[1] on the question of reaction it was shown that the results obtained by titrating the same culture-medium with the same alkaline solution differed very markedly with the indicator employed. For instance, a litre of ordinary meat-infusion nutrient agar-agar required 47 c.c. of a normal caustic alkali solution to neutralize it when phenolphtalein was the indicator used, 28 c.c. when blue litmus was employed, and 5 c.c. when rosolic acid was substituted. It is manifest from this that the actual reactions of media, in the neutralization of which different indicators have been used, may differ very widely from one another, and that the results of cultivation on a medium neutralized by one method are not fairly comparable with those obtained when another indicator has been used. For the sake of uniformity Fuller suggests that bacteriologists should agree upon some one trustworthy method of neutralization and employ it to the exclusion of other methods. He recommends, as the procedure that has given the most satisfactory results in his hands, a modification of Schultz's method, viz., 5 c.c. of the culture-medium are to be mixed with 45 c.c. of distilled water in a porcelain evaporating-dish and boiled for

[1] Fuller: "On the Proper Reaction of Nutrient Media for Bacterial Cultivation," Public Health (Journal of the American Public Health Association), Quarterly Series, 1895, vol. i. p. 381.

three minutes, after which 1 c.c. of phenolphtalein solution[1] is added and titration with the one-twentieth normal caustic alkali solution is quickly made. The neutral point (slightly on the side of alkalinity) is indicated by the appearance of a pink color, the effect of the alkali on the phenolphtalein. From the amount of one-twentieth normal alkali solution needed for 5 c.c. of the medium it is easy to calculate the number of cubic centimetres of the *normal* solution that will be required to neutralize the entire mass.

The phenolphtalein neutral point lies so high, averaging 47 c.c of normal caustic alkali solution per litre for nutrient meat-infusion agar-agar, and 56 c.c. per litre for nutrient gelatin, that it is improbable from experience gained from the older methods that the conditions offered by media *neutral* to this indicator are suitable for the growth of all bacteria, so that with particular species it may be necessary to determine by experiment the degree of deviation from the neutral point that is best suited for development. In Fuller's experience the degree of deviation from the phenolphtalein neutral point that gives in general the best results is represented by from 15 to 20 of his scale—*i. e.*, there should remain enough uncombined acid in a litre of the finished medium to require the *further* addition of caustic alkali to the extent of from 15 to 20 c.c. of a normal solution to bring the reaction of the mass up to the phenolphtalein neutral point. Thus, for example, if upon titration it should be found that to *neutralize* a litre of nutrient meat-infusion gelatin by the phenolphtalein process 55 c.c. of normal caustic alkali solution would be needed, the *amount actually added* would be from 35

[1] A 0.5 per cent. solution of the powder in 50 per cent. alcohol.

to 40 c.c.—*i. e.*, from 15 to 20 c.c. less than the amount needed to bring the reaction up to the neutral point.

Not infrequently the filtered bouillon, neutralized and sterilized, will be seen to contain a fine, flocculent precipitate. This may be due either to excess of alkalinity or to incomplete precipitation of the albumin. The former may be corrected with dilute acetic or hydrochloric acid, and the bouillon again boiled, filtered, and sterilized; or, if due to the latter cause, subsequent boiling and filtration usually result in ridding the bouillon of the precipitate.

Another modification now generally employed is the use of meat-extracts instead of infusion of meat. Almost any of the meat-extracts of commerce answer the purpose, though we usually employ Liebig's. It is used in the strength of from two to four grammes to the litre of water. Peptone and sodium chloride are added as in the bouillon made from meat-infusion. The advantages of meat-extract are: it takes less time; affords a solution of more uniform composition if used in fixed proportions; and in general use gives results that are equally as satisfactory as those obtained from the employment of infusion of meat.

NUTRIENT GELATIN.—For the preparation of gelatin the bouillon is first made in the way given, except that its reaction is corrected after the gelatin has been completely dissolved, which occurs very rapidly in hot bouillon. The reaction of the gelatin of commerce is frequently quite acid, so that a much larger amount of alkali is needed for its neutralization than for other media. It is possible, however, to obtain from the makers an excellent grade of gelatin from

which all acid has been carefully washed.[1] The gelatin is added in the proportion of 10 to 12 per cent. Its complete solution may be accomplished either over a water-bath, in the steam sterilizer, or over a free flame. If the latter method be practised, care must be taken that the mixture is constantly stirred to prevent burning at the bottom and consequent breaking of the flask, if a flask is employed.

For some time it has been our practice to use enamelled iron saucepans, instead of glass flasks, for the purpose of making both gelatin and agar-agar; by this means the free flame may be employed without danger of breaking the vessel, and, with a little care, without burning the media. Under any conditions it is better to protect the bottom of the vessel from the direct action of the flame by the interposition of several layers of wire gauze, a thin sheet of asbestos-board, or an ordinary cast-iron stove-plate.

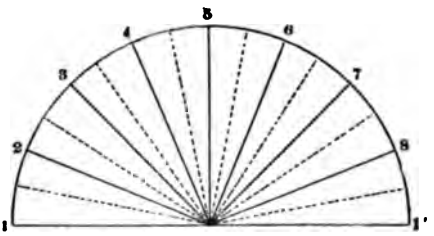
When the gelatin is completely melted it may be filtered through a folded paper filter supported on an ordinary funnel; if solution is complete, this should be very quickly accomplished.

For the filtration of such substances as gelatin and agar-agar it is of much importance to have a properly folded filter. To fold a filter correctly, proceed as follows: a circular piece of filter paper is folded exactly through its centre, forming the fold 1, 1′ (Fig. 16); the end 1 is then folded over to 1′, forming the fold 5; 1 and 1′ are each then brought to 5, thus forming the folds 3 and 7; 1 is then carried to the point 7, and the fold 4 is formed, and by carrying 1′ to 3 the fold 6 is

[1] Hesteberg's acid-free, gold-label gelatin has given us entire satisfaction in this respect.

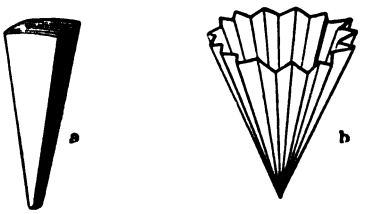
produced; and by bringing 1 to 3 and 1′ to 7 the folds 2 and 8 result.

FIG. 16.



Thus far the ridges of all folds are on the side of the paper next to the table on which we are folding. The paper is now taken up and each space between the seams just produced is to be subdivided by a seam or fold through its centre, as indicated by the dotted lines in Fig. 16, but with the creases on the side *opposite* to that

FIG. 17.



occupied by the creases 1, 2, 3, 4, etc., first made. As each of these folds is made the paper is gradually folded into a wedge-shaped bundle (Fig. 17, *a*), which when opened assumes the form of a properly folded filter (seen in *b*, Fig. 17). Before placing it upon the funnel it is well to go over each crease and see that it is as tightly folded as possible, without tearing it. The

advantage of the folded filter is that by its use a much greater filtering surface is obtained, as it is in contact with the funnel only at the points formed by the ridges, leaving the greater part of the flat surface free for filtration.

The employment of the hot-water funnel, so often recommended, has been dispensed with in this work to a very large extent, for the reason that if solution of the gelatin is complete, filtration is so rapid as not to necessitate the use of an apparatus for maintaining a high temperature. The temperature at which the hot-water funnel retains the gelatin is so high that evaporation and concentration rapidly occur, and in consequence filtration is, as a rule, retarded. The filtration is frequently done in the steam sterilizer; but this too is unnecessary if the gelatin is quite dissolved. At the ordinary temperature of the room, and by the means commonly employed for the filtration of other substances, both gelatin and agar-agar may be rapidly filtered if they are completely dissolved.

It not infrequently occurs that, even under the most careful treatment, the filtered gelatin is not perfectly transparent (the condition to which it must be brought, otherwise it is useless), and clarification becomes necessary. For this purpose the mass must be redissolved, and when at a temperature between 60° and 70° C. an egg, which has been beaten up with about 50 c.c. of water, is added. The whole is then thoroughly mixed together and again brought to the boiling-point, and kept there until coagulation of the albumin occurs. The albumin coagulates as large flocculent masses, and it is better not to break them up if it can be avoided, as when broken up into fine flakes they clog the filter and materially retard filtration.

The practice sometimes recommended of removing these albuminous coagula by first filtering the gelatin through a cloth, and then through paper, is not only superfluous, but in most instances renders the process of filtration much more difficult, because of the disintegration of the masses into finer particles, which have the effect just mentioned, viz., of clogging the filter.

Under no circumstances should a filter be used without first having been moistened with water. If this is not done, the pores of the paper, which are relatively large when in a dry state, when moistened by the gelatin not only diminish in size, but in contracting are often entirely occluded by the finer albuminous flakes which become fixed within them, and filtration practically ceases. The preliminary moistening with water causes diminution of the size of the pores to such an extent that the finer particles of the precipitate *rest on* the surface of the paper, instead of becoming fixed *in its meshes.*

During boiling it is well to filter, from time to time, a few cubic centimetres of the gelatin into a test-tube and boil it over a free flame for a minute or so; in this way one can detect if all the albumin has been coagulated—*i. e.*, if the solution is ready for filtration.

Gelatin should not, as a rule, be boiled more than ten or fifteen minutes at one time, or be left in the steam sterilizer for more than thirty minutes; otherwise its property of solidifying may be impaired.

As soon as the preparation of the gelatin is complete, whether it is retained in the flask into which it has been filtered or decanted into sterilized test-tubes, it should be sterilized in the steam sterilizer on three successive days, for fifteen minutes each day, the mouth

of the flask or the test-tubes containing it having been previously closed with cotton plugs.

NUTRIENT AGAR-AGAR.—The preparation of nutrient agar-agar by the beginner is far too frequently a tedious and time-consuming operation. This is due mainly to lack of patience and to deviation from the rules laid down for the preparation of this medium. If the directions given below for the preparation of nutrient agar-agar be strictly observed, no difficulty whatever should be encountered. Many methods are recommended for its preparation, almost every worker having some slight modification of his own.

The methods that have given us the best results, and from which we have no good grounds for departing, are as follows:

Prepare the bouillon in the usual way. Agar-agar reacts neutral or very slightly alkaline, so that the bouillon may be neutralized before the agar-agar is added. Then add finely chopped or powdered agar-agar in the proportion of 1 to 1.5 per cent. Place the mixture in a porcelain-lined iron vessel, and on the side of the vessel make a mark at the height at which the level of the fluid stands; if a litre of medium is being made, add about 250 to 300 c.c. more of water and allow the mass to boil slowly, occasionally stirring, over a free flame, from one and a half to two hours; or until the excess of water—*i. e.*, the 250 or 300 c.c. that were added—has evaporated. Care must be taken that the mixture does not boil over the sides of the vessel. From time to time observe if the fluid has fallen below its original level; if it has, add water until its volume of 1 litre is restored. At the end of the time given remove the flame and place the vessel

containing the mixture in a large dish of cold water; stir the agar-agar continuously until it has cooled to about 68°–70° C., and then add the white of one egg which has been beaten up in about 50 c.c. of water; or the ordinary dried albumin of commerce may be dissolved in cold water in the proportion of about 10 per cent. and used; the results are equally as good as when eggs are employed. Mix this carefully throughout the agar-agar and allow the mass to boil slowly for about another half-hour, observing all the while the level of the fluid, which should not fall below the litre mark. It is necessary to reduce the temperature of the mass to the point given, 68°–70° C.; otherwise the coagulation of the albumin will occur suddenly in lumps and masses as soon as it is added, and its clearing action will not be uniform. The process of clarification with the egg is purely mechanical; the finer particles, which would otherwise pass through the pores of the filter, being taken up by the albumin as it coagulates and being retained in the coagula.

At the end of one-half hour the boiling mass may be easily and quickly filtered through a heavy, folded paper filter *at the room-temperature;* as a rule, the filtrate is as clear and transparent as agar-agar usually appears.

It may be well to emphasize the fact that for the filtration of agar-agar a hot-water funnel, or any other special device for maintaining the temperature of the mass, is entirely unnecessary. Agar-agar prepared after the methods just given should pass through a properly folded paper filter at the rate of a litre in from twelve to fifteen minutes.

Another plan that insures complete solution of the

agar-agar without causing the precipitates often seen when all the ingredients are added at once and boiled for a long time, is to weigh out the necessary amount of agar-agar, 10 or 15 grammes, and place this in 1300 or 1400 c.c. of water and boil down over a free flame to 1000 c.c. The peptone, salt, and beef-extract are then added and the boiling continued until they are dissolved. The clarification with egg-albumin may then be done, and usually the mass filters quite clear and does not show the presence of precipitates upon cooling. If the mixture is positively alkaline, it is not only cloudy, but it filters with difficulty; if it is acid, it is usually quite clear, and filters more quickly, but, as Schultz has pointed out, it loses at the same time some of its gelatinizing properties. The bouillon should always be neutralized before the agar-agar is added to it; for if the bouillon be acid from the juices of the meat, it robs the agar-agar, under the influence of heat, of part of its gelatinizing power, which will not be regained by subsequent neutralization.

Another method by which agar-agar can be easily and quickly melted is by steam under pressure. If the flask containing the mixture of bouillon and agar-agar be kept in the digester or autoclave for ten minutes with the steam under a pressure of about one atmosphere, as shown by the gauge, the agar-agar will be found at the end of this time completely melted, and filtration may then be accomplished with but little difficulty.

If glycerin is to be added to the agar-agar, it is done after filtration and before sterilization. The nutritive properties of the media for certain organisms, particularly the tubercle bacillus, are increased by the addition of glycerin in the proportion of 5 to 7 per cent.

If after filtration a fine flocculent precipitate is seen, look to the reaction of the medium. If it is quite alkaline, neutralize, boil, and filter again. If the reaction is neutral or only very slightly acid, dissolve and again clarify with egg-albumin by the method given.

The most important feature of all the media, aside from the correct proportion of the ingredients, is their reaction. They must be neutral or very slightly alkaline to litmus. (See remarks on Neutralization of Media.) Only a few organisms develop well on media of an acid reaction. In all of the media mentioned above the meat-extracts now on the market may usually be substituted for the meat itself in preparing the bouillon. They may be employed in the proportion of from two to four grammes to the litre of water.

PREPARATION OF POTATOES.—Potatoes are prepared for use in two ways:

1. They are taken as they come to market—old potatoes being usually recommended—and carefully scrubbed under a water-tap with a stiff brush until all adherent dirt has been removed; "the eyes" and all discolored or decayed parts are carefully removed with a pointed knife. They are then placed in a solution of corrosive sublimate of the strength of 1 : 1000, where they are allowed to remain for twenty minutes; at the end of this time, without rinsing off the sublimate, they are placed in a covered tin bucket with a perforated bottom and sterilized in the steam sterilizer for forty-five minutes. On the second and third days the sterilization is repeated for fifteen to twenty minutes each day. They must not be removed from the sterilizing bucket until sterilization is complete, when they are ready for

use. When prepared in this way they are usually intended to be cut in half, and the cultivation of the organisms is to be conducted upon the flat surfaces of the sections. (Koch's original method.)

This method requires some care to prevent contamination during manipulation. The hand which is to take up the potato from the bucket, which until now has remained covered, is first disinfected in the sublimate solution for ten minutes; the potato is then taken up between the thumb and index finger and severed in two by a knife which has just been sterilized in a free flame until it is quite hot. The knife is passed not quite through the potato, but nearly so. A large glass culture-dish for the reception of the halves of the potato, having been disinfected for twenty minutes with 1 : 1000 sublimate solution and then drained of all adherent solution, should be at hand ready for the potato; the cover is removed, and by twisting the knife gently the halves of the potato may be caused to fall apart in the dish and usually to fall upon their convex surfaces, leaving the flat sections uppermost. The cover of the dish is replaced and the potatoes are ready for inoculation.

2. *Preparation of potatoes for test-tube cultures. Method of Bolton.*[1] If the potatoes are to be employed for test-tube cultures, one simply scrubs off the coarser particles of dirt with water and a brush, and with a cork-borer punches out cylindrical bits of potato which will fit loosely into the test-tubes to be used. On each bit of potato is then to be cut a slanting surface running from about the junction of the first and second thirds of the cylinder to the diagonally opposite end. These cylin-

[1] Medical News, vol. i. of 1887, No. 12, p. 318.

ders of potato are to be left in running water over night, otherwise they will be very much discolored by the sterilization to which they are to be subjected. After being thus washed they are placed in previously prepared test-tubes, one piece in each tube, with the slanting surface up, the cotton plugs of the tubes replaced, and they are then to be sterilized in steam for fifteen to twenty minutes on each of three successive days. Or the entire sterilization may be accomplished in the autoclave, with the steam under a pressure of one atmosphere, by a single exposure of twenty to twenty-five minutes. Potatoes thus prepared have the appearance seen in Fig. 18, except that there is no growth upon the surface as is shown in the cut.

FIG. 18.

Potato in test-tube.

For some purposes potatoes may be advantageously peeled, sliced into discs of about 1 cm. in thickness, and placed in small glass dishes provided with covers, similar to the ordinary Petri dishes. The dish and its contents are then sterilized by steam in the usual way (method suggested by von Esmarch). By this plan a relatively large area for cultivation is obtained.

Potatoes may also be boiled, or steamed, and mashed, and the mass placed in covered dishes, test-tubes, or flasks, and sterilized. By this method one obtains in the mass a mean of the composition of the several potatoes, or bits of potatoes, used in making it, an advantage where uniformity is desired.

Care must be given to the sterilization of potatoes,

because they always have adhering to them the organisms commonly found in the ground, the spores of which are among the most resistant known. The so-called *potato bacillus* is one of this group; it is an organism which is not infrequently more or less of an obstacle to the work of the beginner.

BLOOD-SERUM.—By the original method of preparing blood-serum a great many precautions were taken that have been found unnecessary to the success of the more modern plans.

It is possible to collect serum from small animals and in small quantities under such precautions that it is perhaps not contaminated; but, ordinarily, for laboratory purposes a larger quantity is needed, so that slaughter-houses are the source from which it is usually obtained, and here a certain amount of contamination is unavoidable, though its extent may be limited by proper precautions.

The steps formerly thought essential to the collection of blood and the preparation of serum for culture purposes were about as follows:

The animal from which the blood is to be collected should be drawn up to the ceiling by the hind legs; the head should be held well back, and with one pass of a very sharp knife the throat should be completely cut through. The blood which spurts from the severed vessels should be collected in large glass jars which have been previously cleaned and disinfected, and all traces of the disinfectant removed with alcohol and, finally, with ether. The latter evaporates very quickly and leaves the jar quite dry. The jars should be provided with covers, which close hermetically; these, too, should be carefully disinfected. The best form of vessels for

the purpose is the large museum-jar of about one gallon capacity, which closes by a cover that can be tightly screwed down upon a rubber joint. From two such jarfuls of blood one can recover quite a large quantity of clear serum, ordinarily from 500 to 700 c.c. The jars having been filled with blood, their covers are placed loosely upon them and they are allowed to stand for about fifteen minutes until clotting has begun. At the end of this time a clean glass rod is passed around the edges of the surface of the clot to break up any adhesions to the side of the jar that may have formed, and which would prevent the sinking of the clot to the bottom. The covers are then replaced and tightly clamped in position, and with as little agitation as possible the jars are placed in an ice-chest, where they remain for twenty-four to forty-eight hours. The temperature should, however, not be low enough to prevent coagulation, but should be sufficiently low to interfere with the development of any living organisms that may be present. The temperature of the ordinary domestic refrigerator is sufficient for the purpose. After twenty-four to forty-eight hours the clot will have become firm, and will be seen at the bottom of the jar. Above it is a quantity of dark straw-colored serum. The serum may then be drawn off with a sterilized pipette and placed in tall cylinders that have previously been plugged with cotton wadding and sterilized. After treating all the serum in this way, care having been taken to exclude as much as possible of the coloring-matter of the blood, it may be placed again in the ice-chest for twenty-four hours, during which time the corpuscular elements will sink to the bottom, leaving the supernatant fluid quite clear. This may

then be pipetted off, either into sterilized test-tubes, about 8 c.c. to each tube, or into small sterilized flasks of about 100 c.c. capacity. It is then to be sterilized by the intermittent method *at low temperatures*, viz., for one hour on each of five consecutive days at a temperature of 68°–70° C. During the intervening days it is to be kept at the room-temperature to permit of the development of any spores that may be present into their vegetative forms, in which condition they are killed by an hour's exposure to the temperature of 70° C.

FIG. 19.

Chamber for sterilizing and solidifying blood-serum. (KOCH.)

At the end of this time the serum in the tubes may either be retained as fluid serum or solidified at between 76°–80° C. In solidifying the serum the tubes should be placed in an inclined position, so that as great a surface as possible may be given to the serum. The process of solidification requires constant attention if good

results are to be obtained—*i. e.*, if a translucent, solid medium is the result. If the old, small form of apparatus be employed (Fig. 19), the solidification can be accomplished in a shorter time than if the larger forms commonly employed are used. No definite rule for the time that will be required can be laid down, for this is not constant. If the small solidifying apparatus be used, very good results may be obtained in about two hours at 78° C. It frequently requires a longer time at a higher temperature than has been mentioned. This is especially the case with Löffler's serum-mixture.

The best results are obtained when a low temperature is employed for a long time. Under any circumstances the tubes should be observed from time to time through the glass door or cover with which the solidifying oven is provided, and each time the oven should be slightly jarred with the hand to see if solidification, as indicated by the disappearance of tremors from the serum, is beginning. If the temperature gets too high, or the exposure is too long, an opaque medium results. The temperature to be observed is that of the air inside the chamber, and also that of the water surrounding it. The latter is usually a degree or two higher than the former. The tubes should not rest directly upon the heated bottom or against the heated sides of the chamber, but should lie upon racks of wood or wire, and be protected from the sides by a wire screen or gauze : in this way all the tubes are exposed to about the same temperature. The thermometer which indicates the temperature inside the chamber should not touch the surfaces, but should either be suspended free from above through a cork in the top of the apparatus, if

a large form of apparatus be used; or should lie upon a rack of cork or wood, its bulb being free and a little lower than the other extremity, if the small, old-fashioned apparatus of Koch be employed. The latter form is preferable, as it is more easily managed.

When solidification is complete the tubes are to be retained in the erect position, and, unless they are intended for immediate use, must be prevented from drying. The superfluous ends of the cotton plugs should be burned off, and the mouths of the tubes may then be covered by sterilized rubber caps, or, as Ghriskey suggests, they may be closed with sterilized corks pushed in on top of the cotton plugs. Even with the greatest care it not uncommonly happens that one or two of a lot of tubes thus prepared and protected will become contaminated. This is usually due to spores of moulds that have fallen into the rubber caps or on the cotton plugs during manipulation, and, finding no means of outward growth, have thrown their hyphæ downward through the cotton into the tube, and their spores have fallen on the surface of the serum and developed there.

The foregoing is, in the main, the plan originally recommended by Koch for the preparation of this medium. In recent times, however, particularly since the diagnosis of diphtheria by the method of Löffler has become so general, and large quantities of serum-tubes have been found necessary, a modification has been suggested that has, in this country at least, almost entirely supplanted the method of Koch. The popularity of the Councilman-Mallory method is due to the following facts: by it the serum is more quickly and easily prepared; rigid precautions against contamination

during collection of the serum are not so necessary; and the resulting medium, while not transparent or even translucent (points aimed at in the original method), fully meets all the requirements.

The special points in the method are: the serum is decanted into test-tubes as soon as obtained; it is then firmly coagulated in a slanting position in the *dry-air* sterilizer at from 80° to 90° C.; it is then sterilized in the steam sterilizer at 100° C. on three successive days, as in the case of other culture-media. It may then be protected against evaporation by sterilized rubber caps or sterilized corks in the way already described, and set aside until needed.

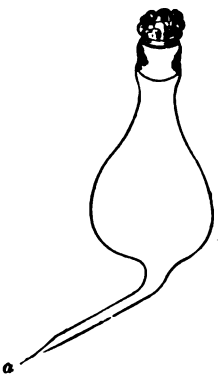
Unless the coagulation in the dry sterilizer be *complete*, the surface of the serum will be found to be blistered and pitted by bubbles and cavities after it has been subjected to the steam sterilization. A similar formation of cavities over the surface of the serum will occur if the temperature of the *hot-air* sterilizer, in which it is solidified, is allowed to get above 90° C., or if it be elevated to this point too quickly.

It is of no special advantage to have the serum clear, as the admixture of blood-coloring-matter does not affect its nutritive properties.

It is often desirable to obtain small quantities of blood-serum under strictly aseptic precautions, and for this purpose Nuttall (*Centralb. für Bakt. u Parasitenkunde*, 1892, Bd. xi. p. 539) suggests a very convenient method. By the use of a sterilized vessel, of the shape shown in Fig. 20, from 10 to 100 c.c. of blood can be collected, and if proper precautions are observed no contamination by bacteria need occur. The collecting bulb is used in the following way: an artery, either femoral or carotid, is

exposed, and around it two ligatures are placed; that distant from the heart is tightened, while the one nearest the heart is left loose; between the latter and the heart the artery is clamped. A small slit is then made in its wall, into which the point *a* of the bulb is introduced and the artery bound tightly around it with the

FIG. 20.



Nuttall's bulb for collecting blood-serum under antiseptic precautions.

hitherto loose ligature; the clamp is removed and the bulb quickly fills with blood. The clamp is now again put in position, the point of the bulb removed and sealed in a gas-flame, the loose ligature tightened, the wound closed, and the bulb containing the blood is set aside in a cool place until coagulation has occurred. The serum is most easily withdrawn from the bulb by means of a pipette, closed above with a cotton-plug, and supplied with a piece of rubber-tubing about one-half metre long, with glass mouth-piece. By holding the pipette in the hand and sucking upon the rubber tube one can more easily direct the point of the pipette than

if it is used in the ordinary way. The bulbs are easily blown, and after having been sealed at the point and plugged with cotton can be kept on hand just as are sterilized test-tubes.

It is sometimes desirable to preserve blood-serum in a fluid state. This can be done by the fractional method of sterilization at low temperatures, already described, or with much less effort, and without the use of heat, by a method that we have found very satisfactory. In the course of Kirschner's investigations chloroform was shown to possess decided disinfectant properties; as it is quite volatile, it is easily got rid of when its disinfectant or antiseptic properties are no longer required. If, therefore, the serum to be preserved be placed in a closely stoppered flask and enough chloroform added to form a thin layer, about 2 mm., on the bottom, the serum may be kept indefinitely without contamination, so long as the chloroform is not permitted to evaporate. When required for use the serum is decanted into test-tubes, which are then placed in a water-bath at about 50° C. until all the chloroform has been driven off; this can be determined by the absence of its characteristic odor. The serum may then be solidified, sterilized by heat, and employed for culture purposes. We have found serum so preserved to answer all requirements as a culture-medium.

SPECIAL MEDIA.—The media just described—bouillon, nutrient gelatin, nutrient agar-agar, potato, and blood-serum—are those in general use in the laboratory for purposes of isolation and study of the ordinary forms of bacteria. For the finer points of differentiation special media have been suggested; a few of them will be mentioned.

Milk. Fresh milk should be allowed to stand over night in an ice-chest, the cream then removed, and the remainder of the milk pipetted into test-tubes, about 8 c.c. to each tube, and sterilized by the intermittent process, at the temperature of steam, for three successive days.

The separation of the cream may be accelerated and rendered more complete if the cylinder containing the milk be placed in the steam sterilizer for fifteen minutes before it is placed in the ice-chest.

The cream is best separated from the milk by the use of a cylindrical vessel with a stopcock at the bottom, by means of which the milk, devoid of cream, may be drawn off. A Chevalier creamometer with a stopcock at the bottom serves the purpose very well. It should be covered while standing.[1]

Milk may be used as a culture-medium without any addition to it, or, before sterilizing, a few drops of litmus tincture may be added, just enough to give it a pale-blue color. By this means it will be seen that different organisms bring about different reactions in the medium: some producing alkalies, which cause the blue color to be intensified; others producing acids, which change it to red; while others bring about neither of these changes. Similarly litmus solution is often added to gelatin and agar-agar for the same purpose.

Milk may also be employed as a solid culture-medium by the addition to it of gelatin or agar-agar in the proportions given for the preparation of ordinary nutrient gelatin or agar-agar. It has, however, in this form

[1] For some time past we have been using what is technically known as "separator milk"— *i. e.*, the fluid left after milk has been deprived of its fat (cream) by centrifugal force.

the disadvantage of not being transparent, and can therefore best be used for the study of those organisms which grow upon the surface of the medium without causing liquefaction.

Nutrient gelatin and agar-agar can also be prepared from neutral milk-whey, obtained from milk after precipitation of the casein.

Dunham's peptone solution. The medium usually known as Dunham's solution is prepared according to the following formula:

Dried peptone	1 part.
Sodium chloride	0.5 "
Distilled water	100 parts.

It is usually of a neutral or slightly alkaline reaction, and neutralization is not, therefore, necessary. It is filtered, decanted into tubes or flasks, and sterilized in the steam sterilizer in the ordinary way. The most common use to which this solution is put is in determining if the organism under consideration possesses the property of producing indol as one of its metabolic products. It is essential for accuracy that the preparation of dried peptone employed should be as nearly chemically pure as is possible, and indeed the other ingredients should be correspondingly free from impurities. Gorini (*Centralblatt für Bakteriologie und Parasitenkunde*, 1893, Bd. xiii. p. 790) calls attention to the fact that impurities in the peptone, particularly the presence of carbohydrates, so interfere with the production of indol by certain bacteria that otherwise produce it, that it is ofttimes impossible, under such circumstances, to obtain the characteristic color-reaction of this body, and where it

is obtained it is always after a much longer time than is the case where peptone free from these substances has been used.

Peckham has also demonstrated that where bacteria have the property of forming indol and also of fermenting carbohydrates, their proteolytic function, as evidenced by the appearance of indol as a product of metabolism, may be completely suppressed by the addition of such fermentable carbohydrates as glucose, saccharose, and lactose to the proteid solution in which they are developing.[1]

Gorini suggests the advisability of testing the purity of all peptone preparations before using them, by means of the reaction that they exhibit with Fehling's alkaline copper solution. Under the influence of this agent pure peptone in solution gives a violet color (the biuret reaction), which remains permanent even after boiling for five minutes. If, instead of a violet color, there appears a red or reddish-yellow precipitate, the peptone should be discarded, as in his experience no indol is produced from peptone giving this reaction. Both the peptone solution and that of the copper (particularly the latter) should be relatively dilute in order for the reaction to be successful.

Peptone rosolic acid solution. Peptone solution containing rosolic acid serves well for the detection of alterations in reaction. It consists of the peptone solution of Dunham, to each 100 c.c. of which 2 c.c. of the following solution are added:

Rosolic acid (coralline)	0.5 gramme.
Alcohol (80 per cent.)	100 c.c.

[1] See Journal of Experimental Medicine, 1897, vol. ii. p. 549.

This is to be boiled, filtered, and decanted into clean, sterilized test-tubes, about 8 to 10 c.c. to each tube. The tubes are then to be sterilized in the usual way by steam. When sterilization is completed and the tubes cooled the solution will be of a very pale rose color, which disappears entirely under the action of acids, but becomes much more intense under the influence of alkalies. We have used this solution for some time for the study of the reactions produced by different organisms, and find it a valuable addition to our means of differentiating bacteria.

Rosolic acid cannot be used in solutions containing glucose, as the reducing action of the latter deprives it of its color.

Lactose litmus-agar, or *litmus-gelatin of Wurtz.* A medium of much use in the differentiation of bacteria is that recommended by Wurtz, consisting of slightly alkaline nutrient agar-agar, to which from 2 to 3 per cent. of lactose and sufficient litmus tincture to give it a pale-blue color have been added. Bacteria capable of causing fermentation of lactose when grown on this medium develop into colonies of a pale-pink color and cause, likewise, a reddening of the surrounding medium, owing to the production of acid as a result of their action upon the lactose; while other bacteria, incapable of such fermentative activities, grow as pale-blue colonies and cause no reddening of the surrounding medium. It is especially useful in the differentiation of the bacillus of typhoid fever, which does not possess the property of bringing about fermentation of lactose, from other organisms that simulate it in many other respects, but which do possess this property.

Its preparation is as follows: to nutrient agar-agar

or gelatin, the alkalinity of which is such that 1 c.c. will require 0.1 c.c. of a 1 : 20 normal sulphuric-acid solution to neutralize it, lactose is added in the proportion of 2 or 3 per cent.; it is then decanted into test-tubes and sterilized in the usual way. When sterilization is complete enough *sterilized* litmus tincture should be added to each tube to give a decided, though not very intense, blue color. This must be done carefully, to avoid contamination of the tubes during manipulation. It is better not to add the litmus tincture before sterilizing the tubes, as its color-characteristics are altered by contact with organic matters under the influence of heat. This medium is used for both test-tube and plate cultivation, just as is ordinary agar-agar and gelatin.

Löffler's blood-serum mixture. Löffler's blood-serum mixture consists of one part of neutral meat-infusion bouillon, containing 1 per cent. of grape-sugar, and three parts of blood-serum. This mixture is placed in test-tubes, sterilized, and solidified in exactly the way given for blood-serum. It requires for its solidification a somewhat higher temperature and a longer exposure to this temperature than does blood-serum to which no bouillon has been added. (See also the Councilman-Mallory method.)

Guarniari's agar-gelatin:

Meat-infusion	950 c.c.
Sodium chloride	5 grammes.
Peptone	25–30 "
Gelatin	40–60 "
Agar-agar	3–4 "
Water	50 c.c.

The special point in the preparation of this medium is its reaction, which should be exactly neutral.

The full list of special media is too extensive to be given in a book of this size. For their description the reader is referred to the current literature. Those that have been given above will suffice for obtaining a clear understanding of the principles of the subject.

NOTE.—The term "meat-infusion" always implies a watery extract of meat made by mixing 500 grammes of finely chopped lean meat and 1 litre of water, and allowing them to stand in a cool place for twenty-four hours. At the end of this time the fluid portion is strained off through a coarse towel, and this is what is meant by the "meat-infusion."

CHAPTER VI.

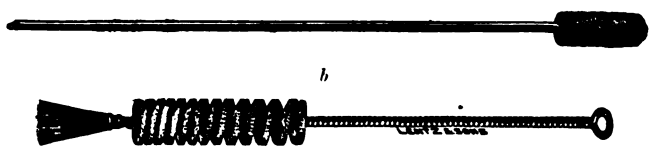
Preparation of the tubes, flasks, etc., in which the media are to be preserved.

WHILE the media are in course of preparation it is well to get the test-tubes and flasks ready for their reception, and it is essential that they should be as clean as it is possible to make them. For this purpose it is advisable that both new tubes and those which have previously been used should be boiled for about thirty to forty-five minutes in a 2 to 3 per cent. solution of common soda; it is not necessary to be exact as to strength, but it should not be weaker than this. At the end of this time they are to be carefully swabbed out with a cylindrical bristle brush, preferably one with a reed handle (Fig. 21, *a*), as those with wire handles

FIG. 21.

a

b



Brushes for cleaning test-tubes.

are apt to break through the bottoms of the tubes, though Messrs. Lentz & Sons, of this city, have in large part eliminated this objection from the wire-handle brush depicted in Fig. 21, *b*. All traces of adherent

material should be carefully removed. When the tubes are quite clean they may be rinsed in a warm solution of commercial hydrochloric acid of the strength of about 1 per cent. This is to remove the alkali. They are then to be thoroughly rinsed in clear, running water, and stood top down until the water has drained from them. When dry they are to be plugged with raw cotton; this requires a little practice before it can be properly done. The cotton should be introduced into the mouths of the tubes in such a way that no cracks or creases exist. The plug should fit neither too tightly nor too loosely, but should be just firmly enough in position to sustain the weight of the tube into which it is placed when held up by the portion which projects from and overhangs the mouth of the tube. The tubes thus plugged are now to be placed upright in a wire basket and heated for one hour in the hot-air sterilizer at a temperature of about 150° C. A very good guide for this process of sterilization is to observe the tubes from time to time, and as soon as the cotton has become a very light-brown color, not deeper than a dark-cream tint, to consider sterilization complete. The tubes are then removed and allowed to cool.

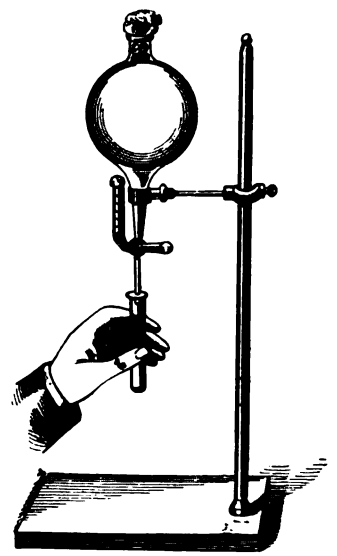
The cotton used for this purpose should be the ordinary cotton batting of the shops, and not absorbent cotton; the latter becomes too tightly packed, and is, moreover, much too expensive for this purpose.

Care should be taken not to burn the cotton, otherwise the tubes will become coated with a dark-colored, empyreumatic, oily deposit, which necessitates recleansing.

FILLING THE TUBES.—When the tubes are cold they may be filled. This is best accomplished by the

use of a separating funnel, such as is shown in Fig. 22. The liquefied medium is poured into this funnel, which has been carefully washed, and by pressing the pinchcock with which the funnel is provided the desired amount of material (5–10 c.c.) may be allowed to flow into the tubes held under its opening. It is not necessary to sterilize the funnel, for the medium is to be subjected to this process as soon as it is in the test-tubes.

FIG. 22.



Funnel for filling tubes with culture-media.

Care should be taken that none of the medium is dropped upon the mouth of the test-tube, otherwise the cotton plug becomes adherent to it, and is not only difficult to remove, but presents a very untidy appearance and interferes materially with the manipulations.

As soon as the tubes have been filled they are to be sterilized in the steam sterilizer for fifteen minutes on each of three successive days. During the intervening days they may be kept at the ordinary room-temperature.

When sterilization is complete and the medium in the tubes is still liquid, some of them may be placed in a slanting position, at an angle of about ten degrees with the surface on which they rest, and the medium allowed to solidify in this position. These are for the so-called slant-cultures. The remainder may solidify in the erect position; these serve for making plates.

For Esmarch tubes not more than 5 c.c. of material should be placed in each tube, as more than this renders it difficult to distribute the gelatin evenly over the inner surface of the tubes when they are rolled.

CHAPTER VII.

Technique of making plates—Esmarch tubes, Petri plates, etc.

PLATES.—The plate method can be employed with both agar-agar and gelatin. It cannot be practised with blood-serum, because the serum when once solidified cannot be again liquefied.

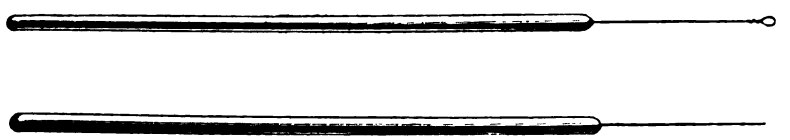
Plates are usually referred to as "a set." This term implies three individual plates, each representing a mixture of organisms in a higher state of dilution. The first plate is known usually as "the original," or "plate No. 1," the first dilution from this as "plate No. 2," and the second as "plate No. 3."

In the preparation of a set of plates the following are the steps to be observed:

Three tubes, each containing from 7 to 9 c.c. of gelatin or agar-agar, are placed in a warm water-bath until the medium has become liquid. If agar-agar is employed, this is accomplished at the boiling-point of water; if gelatin is used, a much lower temperature suffices (35°–40° C.). When liquefaction is complete the temperature of the water, in the case of agar-agar, must be reduced to 41°–42° C., at which temperature the agar-agar remains liquid, and the organisms may be introduced into it without fear of destroying their vitality. The medium being now liquid and of the proper temperature, a very small portion of the mixture of organisms to be studied is taken up with a sterilized

platinum wire (Fig. 23, *a*) about 5 cm. long, twisted into a small loop at one end and fused into a bit of glass rod, which serves as a handle, at the other extremity. This loop is one of the most useful of bacteriological instruments, as there is hardly a manipulation into which it does not enter. Under no circumstances is it to be employed without having been passed through a gas-flame until quite hot, for the purpose of sterilization. One should form a habit

FIG. 23.



a

b

Looped and straight platinum wires in glass handles.

of never taking up one of these platinum-wire needles, as they are called, for they are curved and straight (Fig. 23, *b*) as well as looped, without passing it through a flame; and the sooner the beginner learns to do this as a reflex action, the sooner does he eliminate one of the possible sources of error in his work. It must be remembered, though, that it should not be used when hot, otherwise the organisms taken upon it will be killed by the high temperature; after sterilization in the flame one waits for a few seconds until it is cool before using.

A minute portion of the material under consideration is transferred with the sterilized loop into tube No. 1, "the original," where it is thoroughly disintegrated by gently rubbing it against the sides of the tube. The more carefully this is done the more uniform will be

the distribution of the organisms and the better the results. The loop is then again sterilized and three of its loopfuls are passed, *without touching the sides of the tube*, from "the original" into tube No. 2, where they are carefully mixed. Again the loop is sterilized, and again three dips are made from tube No. 2 into tube No. 3. This completes the dilution. The loop is now sterilized before laying it aside.

FIG. 24.

Levelling-tripod with glass chamber for plates.

During this manipulation, which must be done quickly if agar-agar be employed, the temperature of the water in the bath in which the tubes stand should never be lower than 39° C., and never higher than 43° C. If it falls below 38° C., the agar-agar solidifies, and can only be redissolved at a temperature that would be destructive to the organisms which may have been introduced into the tubes. This is not of much moment with gelatin, since it may readily be

redissolved at a temperature not detrimental to the organisms with which the tubes may have been inoculated.

THE COOLING-STAGE AND LEVELLING-TRIPOD.—While the medium of which the plates are to be made is melting it is well to arrange the cooling-stage (Fig. 24) upon which the gelatin or agar-agar is to be subsequently solidified.

This stage consists of a glass dish filled with ice-water and covered with a ground-glass plate, which in turn has a dome-shaped cover. The dish rests upon a tripod which can be brought to an exact level, as indicated by the spirit-level, by raising or lowering its legs by means of thumb-screws, with which they are provided. Three stages are usually employed. When ready for use they should be exactly level.

THE GLASS PLATES.—On each of the stages is to be placed a glass plate upon which the liquefied gelatin or

FIG. 25.

Russia iron box for holding plates, etc., during sterilization in dry heat.

agar-agar is to be poured and allowed to solidify. It is, therefore, necessary that the plates should not only be sterile when placed upon the stages, but that during manipulation they should be carefully protected by a cover against dust and bacteria from without.

A number of plates at a time are usually sterilized in the dry sterilizer at a temperature of 150° to 180° C. for one hour. During sterilization and until used they are retained in an iron box (Fig. 25), which is especially designed for the purpose. They should never be placed upon the stage until cold; otherwise they crack.

When the plates which have been placed upon the stages are quite cold the melted gelatin or agar-agar in the tubes which represent the three dilutions should be poured upon them, each tube being emptied upon a separate plate. If the medium is quite fluid, it spreads over the surface of the plates in a thin, even layer. Sometimes it may be more evenly spread as it flows from the tube by the aid of a sterilized glass rod.

FIG. 26.

Glass benches for supporting plates.

As the content of each tube is emptied the cover of the cooling-stage is quickly replaced and the plate allowed to stand until the gelatin or agar-agar is quite solid. This takes longer with gelatin than with agar. When the medium is quite solid these plates are placed upon glass benches (Fig. 26), each labelled with the number of the plate in the series of dilutions. The benches, with the plates upon them, are then piled one above the other in a "culture-dish," in which the plates are to be kept during the growth of the bacteria. The benches

are sterilized before using, in the same manner as plates.

CULTURE-DISH.—This dish, which is about 22 cm. in diameter and has vertical sides about 6 cm. in height, is provided with a cover of exactly the same shape, but of a little larger diameter. This cover, when placed upon the dish containing the plates, prevents the access of dust. Prior to using, the dish and cover should have been disinfected for one-half hour with 1 : 1000 sublimate, and all the solution allowed to drain from them.

A disk of sterilized filter-paper moistened with sterilized water is sometimes placed in the bottom of the culture-dish to prevent drying of the medium. This is not often necessary, however, as the plates can usually be dispensed with before evaporation has progressed very far.

If agar-agar be employed, the dish and its contents may be kept at a temperature of 37°–38° C.; if gelatin, the temperature at which the plates are to be maintained should not be over 22° C., otherwise the gelatin liquefies and the plates are rendered useless.

When development has occurred the object of the dilution will be easily seen, and the various species of bacteria in the mixture will be recognized by differences in the character of the colonies growing from them.

This, in short, is the plate method of Koch for the separation of the individual species contained in a mixture of bacteria. Many modifications of this method exist; all, however, are based upon the same principles. The modifications have for their object the accomplishment of the same end, but with a smaller armamentarium of apparatus; according to individual

preference, the one or the other of these modifications has supplanted the original plate method as practised and recommended by Koch.

Petri's Modification of the Plate Method.—The modification which approaches nearest to the original method, and at the same time lessens very materially the number of steps in the process, is that suggested by Petri. It consists in substituting for the plates small, round, double glass dishes, having about the same surface-area as the plates (Fig. 27). The liquid medium is poured directly into these little dishes and their covers replaced; they are then set aside for observation. In all other respects the process is the same as Koch's original method. Petri's dishes are about 8

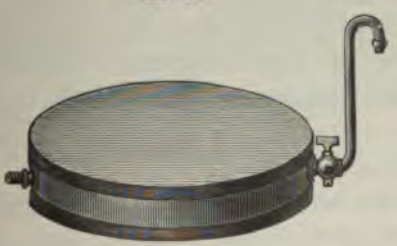
Fig. 27.

Petri double dish, now generally used instead of plates.

cm. in diameter and about 1.5 to 2 cm. in height, the sides being vertical. They may readily be sterilized by either hot-air or steam. They are very useful for this work, as they do away with the necessity for the cooling-stage and levelling-tripod, though in warm weather the cooling-stage may be used to hasten the solidification of gelatin. A cooling-stage of very convenient design for use with these dishes consists of a closed, flat metal box, either of copper or block tin, and round or square in shape, so arranged that it can be filled with cold water, or that cold water can constantly be

passed through it by means of a rubber tube connected with a spigot. The inlet for the water should be just above the bottom of the box, and the outlet just beneath the top and slightly turned upward and then downward, so as to insure filling the space with water. The box should be sufficiently strong to resist the pressure of the water. A convenient size is from 20 to 25 cm. in diameter, and about 1.5 to 2 cm. high. It is simple in construction, and can be made by any copper-spinner. An idea of its construction is given in Fig. 28.

FIG. 28.



Metal cooling-stage.

When gelatin or agar-agar is to be cooled it is only necessary to place the dishes containing it on top of this box and keep cold water circulating through it.

ESMARCH TUBES.—The modification of Koch's method which insures the greatest security from contamination by extraneous organisms and requires the least amount of apparatus is that suggested by v. Esmarch. It differs from the other methods thus: the dilutions having been prepared in tubes containing a smaller amount of medium than usual—as a rule, not more than 5 to 6 c.c.—are, instead of being poured upon plates or into dishes, spread over the inner surface of

the tubes containing them, and, without removing the cotton plugs, solidified in this position. The tubes then present a thin cylindrical lining of gelatin or agar-agar, upon which the colonies develop. In all other respects the conditions for the growth of the organisms are the same as in flat plates.

Esmarch directs that after completion of the dilutions the tops of the cotton plugs in the test-tubes should be cut off flush with the mouths of the tubes and sterilized rubber caps be placed over them. They are then to be held in a horizontal position and twisted between the fingers upon their long axis under ice-water. The gelatin becomes solidified thereby and adheres to the sides of the tube. When the gelatin is quite hard the tubes are removed from the water, wiped dry, the rubber caps removed, and the tubes set aside for observation.

For some time past we have deviated from the direction given by v. Esmarch for this part of his method, and instead of rolling the tubes under ice-water, we roll them upon a block of ice (Fig. 29), after the method devised by Booker in 1887 in the Pathological Laboratory of the Johns Hopkins University. In this method a small block of ice only is needed. It is levelled and held in position by being placed upon a towel in a dish. A horizontal groove is melted in the upper surface of the ice with a test-tube of hot water. The tubes to be rolled are then held in an almost—*not quite*—horizontal position and twisted between the fingers until the sides are moistened by the contents to within about 1 cm. of the cotton plug, care being taken that the gelatin *does not touch* the cotton; otherwise the latter becomes adherent to the sides of the tube and is difficult

to remove. The tube is then placed in the groove in the ice and rolled, neither rubber cap nor cutting off of the cotton plug being necessary.

FIG. 29.

Demonstrating Booker's method of rolling Esmarch tubes on a block of ice.

The advantages of this process over that followed by v. Esmarch are that it requires less time, is cleaner, no rubber caps are needed, the rolled tubes are more uniform, and the gelatin does not touch the cotton plug, as is always the case in tubes rolled under water, because of the impossibility of keeping them at one level.

There is an erroneous impression that Esmarch tubes are not a success when made from ordinary nutrient agar-agar because of the tendency of this medium to shrink and fall to the bottom of the tube. This slipping down of the agar-agar is due to the water squeezed from it during solidification getting between the medium and the walls of the tube. This can easily be overcome by allowing the rolled tubes to remain in a nearly horizontal position for twenty-four hours after rolling them, the mouth of the tube being about 1 cm. higher

than the bottom. During this time the margin of the agar-agar nearest the cotton plug dries and becomes adherent to the walls of the tube, while the water collects at the most dependent point—*i. e.*, the bottom of the tubes. After this they may be retained in the upright position without danger of the agar-agar slipping down. We have employed this method for several years with entire satisfaction. In all these manipulations, if the dilutions of the number of organisms have been properly conducted, the results will be the same. The original plate or tube, as a rule, will be of no use because of the great number of colonies in it; plate or tube No. 2 may be of service; but plate or tube No. 3 will usually contain the organisms in such small numbers that there will be nothing to prevent the characteristic development of the colonies originating from them.

For reasons of economy the "original," tube No. 1, is sometimes substituted by a tube containing normal salt-solution (0.6 to 0.7 per cent. of sodium chloride in water), which is thrown aside as soon as the dilutions are completed, and only plates or tubes Nos. 2 and 3 are made.

Another method for the separation of bacteria and their isolation as single colonies consists in the making of dilutions upon the surface of solid media, such as potato, coagulated blood-serum, agar-agar, and gelatin. In pursuance of this method one selects a number of tubes containing the medium set in a slanting position. With a platinum needle a bit of the substance to be studied is smeared upon tube No. 1; without sterilizing the needle it is passed in succession over the surface of the medium in tubes Nos. 2, 3, 4, etc. When de-

velopment has occurred essentially the same conditions as regards separation of the colonies will be found as when plates are poured. If a slanted medium be employed, about the most dependent angle of which water of condensation has accumulated, as blood-serum, agar-agar, and potato, the dilutions may be made in this fluid, and this is then to be carefully smeared over the solid surface of the medium. The tubes thus treated should be kept in an upright position to prevent the fluid flowing over the surface. When sufficiently developed, single colonies may be isolated with comparative ease from tubes prepared in this manner. (See also method for the isolation of *bacillus diphtheriæ* on blood-serum.)

CHAPTER VIII.

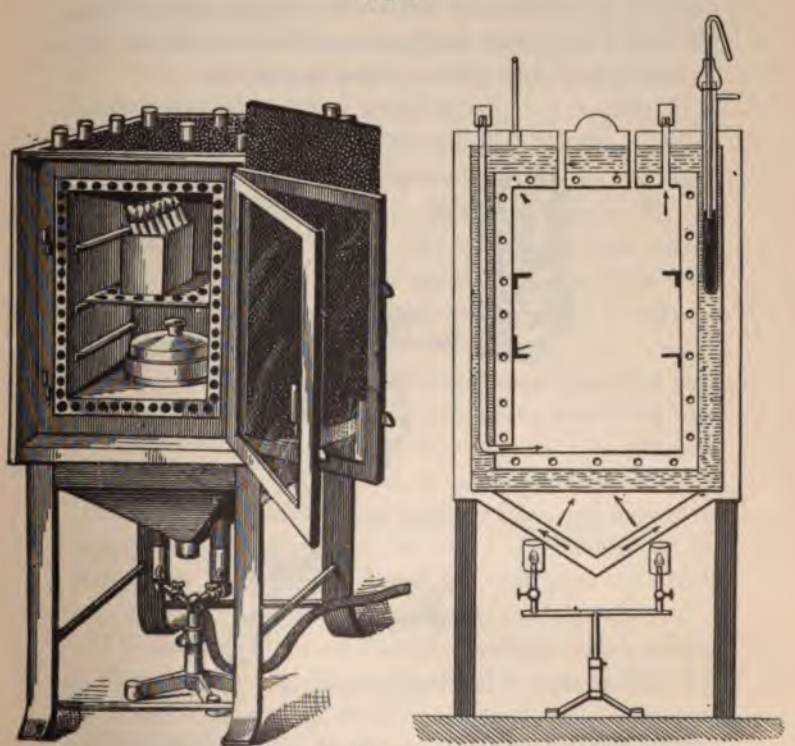
The incubating-oven—Gas-pressure regulator—Thermo-regulator—The safety burner employed in heating the incubator.

THE INCUBATOR.—When the plates have been made it must be borne in mind that for the development of certain forms of bacteria a higher temperature is necessary than for the growth of others. The pathogenic or disease-producing organisms grow more luxuriantly at the temperature of the human body (37.5° C.) than at lower temperatures; whereas for the ordinary saprophytic forms almost any temperature between 18° and 37° C. is suitable. It therefore becomes necessary to provide a place in which a constant temperature favorable to the growth of the pathogenic organisms can be maintained. For this purpose a number of different forms of apparatus have been devised. They are all based upon the same principles, however, and a general description of the essential points involved in their construction will be all that is needed here.

The apparatus known as thermostat, incubator, or brooding-oven, is a copper chamber (Fig. 30) with double walls, the space between which is filled with water. The incubating-chamber has a closely fitting double door, inside of which is usually a door of glass through which the contents of the chamber may be inspected without actually opening it. The whole apparatus is encased in either asbestos-boards or thick felt,

to prevent radiation of heat and consequent fluctuations in temperature. In the top of the chamber is a small opening through which a thermometer projects into its interior. At either corner, leading into the space containing the water, are other openings for the reception

FIG. 30.



Incubator used in bacteriological work.

of another thermometer and a thermo-regulator, and for refilling the apparatus as the water evaporates. On the side is a water-gauge for showing the level of the water

between the walls. The object of the water-chamber, which is formed by the double-wall arrangement, is to insure, by means of the warmed water, an equable temperature in all parts of the apparatus—at the top as well as at the sides, back, and bottom; the apparatus should be kept filled with water, otherwise the purpose for which it is constructed will not be accomplished. When the chamber between the walls is filled with water heat is supplied by a gas-flame placed beneath it.

FIG. 31.

Koch's safety burner.

The burner employed in heating the incubator was originally devised by Koch, and is known as "Koch's safety burner" (Fig. 31). It is a Bunsen burner provided with an arrangement for automatically turning off the gas-supply and thus preventing accidents should

the flame become extinguished at a time when no one is near. The gas-cock by which the gas is turned on and off is provided with a long arm which is weighted, and which, when the gas is turned on and *burning*, rests upon an arm attached to the side of a revolving, horizontal disk that is connected with the free ends of two metal spirals which are fixed by their other ends in opposite directions on either side of the flame and heated by it. If by draughts or any other accident the flame becomes extinguished, the metal spirals cool, and in cooling contract, twist the horizontal disk in the opposite direction, and allow the weighted arm of the gas-cock to fall. By its falling the gas-supply is turned off.

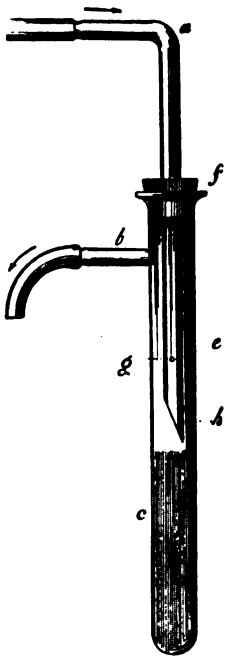
THERMO-REGULATORS.—The regulation and maintenance of the proper temperature within the incubator are accomplished by the employment of an automatic thermo-regulator.

The common form of thermo-regulator used for this purpose is constructed upon principles involving the expansion and contraction of fluids under the influence of heat and cold. By means of this expansion and contraction the amount of gas passing from the source of supply to the burner may be either diminished or increased as the temperature of the substance in which the regulator is placed either rises or falls.

The simplest form of thermo-regulator which serves to illustrate the principles involved is seen in Fig. 32. It consists of a glass cylinder, *e*, having a communicating branch tube *b*, and rubber stopper *f*, through which projects the bent tube *a*. The tube *a* is ground to a slanting point at the extremity which projects into the tube *e*, and is provided a short distance above this point with a capillary opening, *g*, in one of its sides.

When ready for use the cylinder *e* is filled with mercury up to about the level shown in the figure. It is then allowed to stand, or is suspended, in the bath the temperature of which it is to regulate. The rubber tubing coming from the gas-supply is attached to the

FIG. 32.



Mercurial thermo-regulator.

outer end of the glass tube *a*, and the tube going to the burner is slipped over the branch tube *b*. The gas is turned on and the burner lighted and placed under the bath. The gas now streams through the tube *a* into the cylinder *e* and out at *b* to the burner; but as the tem-

perature of the bath rises the mercury contained in the cylinder *e*, under the influence of the elevation of temperature, begins to expand, and, as a continuous rise in temperature proceeds, the expansion of the mercury accompanies it and gradually closes the slanting opening *h* of tube *a*. In this way the supply of gas becomes diminished and the rise in temperature of the bath will be less rapid, until finally the opening at *h* will be closed entirely, when the supply of gas to the burner will now be limited to that passing through the capillary opening *g*. This is not sufficient to maintain the highest temperature reached, and as cooling begins a gradual contraction of the mercury occurs until there is again an outflow of gas from the opening *h*, when the temperature again rises. This contraction and expansion of the mercury in the regulator continues until eventually a point is reached at which its position in the cylinder *e* allows of the passage of just enough gas from the opening *h* to maintain a constant temperature and, therefore, a constant degree of expansion of the mercury in the tube *e*. This, in short, is the principle on which thermo-regulators are constructed; but it must be borne in mind that a great deal of detail exists in the construction of an accurate instrument. The number of different forms of this apparatus is comparatively large, and each form has its special merits.

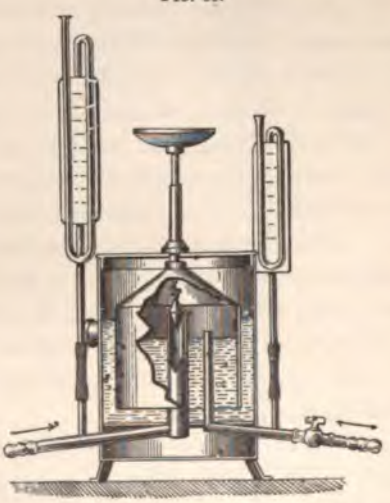
The value—that is, the delicacy—of the thermo-regulator depends upon a number of factors, all of which it would be useless to describe in a book of this kind; but in general it may be said that the essential points to be observed in selecting a thermo-regulator depend in the main upon the temperatures at which it is to be used. For low temperatures, regulators containing such fluids

as ether, alcohol, and calcium chloride solution, which expand and contract rapidly and regularly under slight variations in temperature, are commonly employed; whereas for temperatures approaching the boiling-point of water mercury is most frequently used.

The temperature of the incubator is to be regulated, then, by the use of some such form of apparatus as that just described. It should be of sufficient delicacy to prevent a fluctuation of more than 0.2° C. in the temperature of the air within the chamber of the apparatus.

Gas-pressure Regulators.—A gas-pressure regulator is not rarely intervened between the gas-supply

Fig. 33.



Moitessier's gas-pressure regulator.

and the thermo-regulator. This apparatus has for its object the maintenance of a constant pressure of the gas going to the thermo-regulator. There are several

forms of regulator in use, but they do not accomplish the object for which they are designed.

The instrument most commonly employed, the apparatus of Moitessier (Fig. 33), is based on somewhat the same principles as the large regulators seen at the manufactories of illuminating-gas. Such apparatus act very well when employed on the large scale, as one sees them at the gas-works; but when applied to the limited and sudden fluctuations seen in the gas coming from an ordinary gas-cock are practically useless. They are too gross in their construction, and act only under comparatively great and gradual fluctuations in pressure. If a good form of thermo-regulator be employed, there is no necessity for the use of any of the pressure-regulators thus far introduced.

CHAPTER IX.

The study of colonies—Their naked-eye peculiarities and their appearance under different conditions—Differences in the structure of colonies from different species of bacteria—Stab-cultures—Slant-cultures.

THE plates of agar-agar which have been prepared from a mixture of organisms and have been placed in the incubator, and those of gelatin which have been maintained at the ordinary temperature of the room, are usually ready for examination after from twenty-four to forty-eight hours. They will be found marked here and there by small points or little islands of more or less opaque appearance. In some instances these will be so transparent that it is with difficulty one can see them with the naked eye. Again, they may be of a dense, opaque appearance; at one time sharply circumscribed and round, again irregular in their outline; here a point will present one color, there perhaps another. On gelatin some of the points will be seen to be lying on the surface of the medium, others will have sunk into little depressions, while at still other points the clear gelatin will be marked by more or less saucer-shaped pits containing opaque fluid.

Place the plate containing these points upon the stage of a microscope and examine them with a low-power objective, and again differences will be observed. Some of these minute points will be finely granular, others coarsely so; some will present a radiated appearance, while a neighbor may be concentrically arranged:

here nothing particularly characteristic will present, there the point may resolve itself into a mass having somewhat the appearance of a little pellicle of raw cotton. All these differences, and many more, aid us in saying that these objects must be different in their nature. With a pointed platinum needle take up a bit of one of these small islands, prepare it for microscopic examination (see chapter on Stained Cover-slip Preparations), and examine it under the high-power oil-immersion objective, with access of the greatest amount of light afforded by the illuminator of the microscope. The preparation will be seen to be made up entirely of bodies of the same shape; they will all be spheres, or ovals, or rods, but not a mixture of these forms, if proper care in the manipulation has been taken. Examine in the same way a neighboring spot which possesses different naked-eye appearances, and it will often be found to consist of bodies of an entirely different appearance from those seen in the first preparation.

These spots or islands on the surface of the plates are colonies of bacteria, differing severally, not only in their gross appearances, the one from the other, but, as our cover-slip preparations show, in the morphological characteristics of the individual organisms composing them.

If from one of these colonies a second set of plates be prepared, the peculiarities which were first observed in it will be reproduced in all of the new colonies which develop; each will be found to consist of the same organisms as the colony from which the plates were made. In other words, these peculiarities are constant under like conditions.

The appearance of the colonies developing from all organisms is regulated by their location in the

medium in which they are growing. When deep down in the medium they are usually round, oval, or lozenge-shaped; whereas when on the surface of the gelatin or agar they may take quite a different form. This is purely a mechanical effect due to the pressure of, or resistance offered by, the medium surrounding them, and is always to be borne in mind, otherwise errors are apt to arise.

PURE CULTURES.—If from one of these small colonies a bit be taken upon the point of a *sterilized* platinum needle and introduced into a tube of sterilized gelatin or agar-agar, the growth that results will be what is known as a "pure culture," the condition to which all organisms must be brought before a systematic study of their many peculiarities is begun. Sometimes several series of plates are necessary before the organisms can be obtained pure, but by patiently following this plan the results will ultimately be satisfactory.

TEST-TUBE CULTURES; STAB-CULTURES; SMEAR-CULTURES.—After separating the organisms the one from the other by the plate method just described, they must be isolated from the plates as pure stab- or smear-cultures.

This is done in the following way: decide upon the colony from which the pure culture is to be made. Select preferably a small colony and one as widely separated from other colonies as possible. Sterilize in a gas-flame a straight platinum-wire needle. The glass handle of the needle should be drawn through the flame as well as the needle itself, otherwise contamination from this source may occur. When it is cool, which is in five or ten seconds, take up carefully a portion of the colony. Guard against touching *anything but the colony.* If

during manipulation the needle touches *anything else whatever* than the colony from which the culture is to be made, it must be sterilized again. This holds not only for the time before touching the colony, but also during its passage into the test-tube from the colony; otherwise there is no guarantee that the growth resulting from the inoculation of this bit of colony into a fresh sterile medium will be pure.

In the meantime have in the other hand a test-tube of sterile medium: gelatin, agar-agar, or potato. This tube is held across the palm of the hand in an almost horizontal position with its mouth pointing out between the thumb and index-finger and its contents toward the body of the worker. With the disengaged fingers of the other hand holding the needle the cotton plug is removed from the tube by a twisting motion and placed between the index and second fingers of the hand holding the tube, in such a way that the portion of the plug which fits into the mouth of the test-tube looks toward the dorsal surface of the hand and *does not touch any portion of the hand;* this is accomplished by placing *only the overhanging* portion of the plug between the fingers. The needle containing the bit of colony is now to be thrust into the medium in the tube if a stab-culture is desired, or rubbed gently over its surface if a smear-culture is to be made. The needle is then withdrawn, the cotton plug replaced, *and the needle sterilized* before it is laid down. Neither the needle nor its handle should touch the inner sides of the test-tube if it can be avoided. The tube is then labelled and set aside for observation. The growth which appears in the tube after twenty-four to thirty-six hours will be a pure culture of the organisms of which the colony was composed.

Cultures of this form are not only useful as a means of preserving the different organisms with which we may be working, but serve also to bring out certain

FIG. 34.

Series of stab-cultures in gelatin, showing modes of growth of different species of bacteria.

characteristics of different organisms when grown in this way.

If gelatin be employed and the organism which has been introduced into it possesses the power of bringing about liquefaction, it will soon be discovered that the

mode of liquefaction differs with different organisms. Some bacteria cause a liquefaction which spreads across the whole upper surface of the gelatin and continues gradually downward; with others it occurs in a funnel-shape, the broad end of the funnel being uppermost and the point downward, corresponding to the track of the needle; at times a stocking- or sac-like liquefaction may be noticed. (See Fig. 34.)

NOTE.—Obtain a number of organisms from different sources in pure cultures by the method given. Plant them as pure cultures, all at the same time, in gelatin—preferably gelatin of the same making—retain them under the same conditions of temperature, and sketch the finer differences in the way in which liquefaction occurs.

CHAPTER X.

Methods of staining—Solutions employed—Preparation and staining of cover-slips—Preparation of tissues for section-cutting—Staining of tissues—Special staining-methods.

A COMPLETE list of solutions and methods that are recommended for the staining of bacteria is not essential to the work of the beginner, so that only those which are of the most common application will be given in this book. In general, it suffices to say that bacteria stain best with watery solutions of the basic aniline dyes, and of these, fuchsin, gentian-violet, and methylene-blue are those most frequently employed.

In practical work bacteria are either dried upon cover-slips and then stained, or stained in sections of tissues in which they have been deposited during the course of disease. In both processes the essential point to be borne in mind is that the bacteria, because of their microscopic dimensions, require to be more conspicuously stained than the surrounding materials upon the cover-slips or in the sections, otherwise their recognition is a matter of the greatest difficulty, if not of impossibility. For this reason, especially in section-staining, it frequently becomes necessary to decolorize the tissues after removing them from the staining-solutions, in order to render the bacteria more prominent, and for this purpose special methods, which provide for decolorization of the tissues without robbing the bacteria of their color, are employed. The ordinary method of cover-slip examination of bac-

teria, constantly in use in these studies, is performed in the following way:

COVER-SLIP PREPARATIONS.—In order that the distribution of the organisms upon the cover-slips may be uniform and in as thin a layer as possible it is essential that the slips should be clean and free from grease. For cleansing the slips several methods may be employed.

The simplest plan with new cover-slips is to immerse them for a few hours in strong nitric acid, after which they are rinsed in water, then in alcohol, then ether, and, finally, they may be kept in alcohol to which a little ammonia has been added. When about to be used they should be wiped dry with a clean cotton or silk handkerchief.

If the slips have been previously used, boiling in strong soap solution, followed by rinsing in clean warm water, and then treating as above, renders them clean enough for ordinary purposes.

A method commonly employed is to remove all coarse adherent matter from slips and slides by allowing them to remain for a time in strong nitric or sulphuric acid. They are removed from the acid after several days, rinsed in water, and treated as above. Knauer has recently suggested the boiling of soiled cover-slips and slides for from twenty to thirty minutes in a 10 per cent. watery solution of lysol, after which they are to be carefully rinsed in water until all trace of the lysol has disappeared. They are then to be wiped dry with a clean handkerchief.

Löffler's method, which provides for the complete noval of all grease, is to warm the cover-slips in con-
rated sulphuric acid for a time and then rinse them

in water, after which they are kept in a mixture of equal parts of alcohol and ammonia. They are to be dried on a cloth from which all fat has been extracted.

Steps in making the preparations. Place upon the centre of one of the clean, dry cover-slips a very small drop of distilled water or physiological salt-solution. With a platinum needle, which has been sterilized in a gas-flame *just before using* and allowed to cool, take up a very small portion of the colony to be examined and mix it carefully with the drop on the slip until there exists a very thin homogeneous film over the larger part of the surface. This is to be dried upon the slip by either allowing it to remain upon the table in the horizontal position under a cover, to protect it from dust, or by holding it *between the fingers* (*not with forceps*), at some distance above a gas-flame, until it is quite dry. If held with the forceps over the flame at this stage, too much heat may be unconsciously applied, and the morphology of the organisms in the preparation distorted. When held between the fingers with the thin layer of bacteria *away from the flame* no such accident is likely to occur. When the whole pellicle is completely dried the slip is to be taken up with forceps, and, holding the side upon which the bacteria are deposited away from the direct action of the flame, it is to be passed through the flame three times, a little more than one second being allowed for each transit. Unless the preliminary drying at the low temperature has been complete, the preparation will be rendered worthless by the subsequent "fixing" at the higher temperature, for the reason that the protoplasm of bacteria when moist coagulates at these temperatures, and in doing so the normal outline of the cells is

altered. If carefully dried before fixing, this does not occur and the morphology of the organism remains unchanged.

A better plan for the process of fixing is to employ a copper plate about 35 cm. long by 10 cm. wide by 0.3 cm. thick. This plate is laid upon an iron tripod and a small gas-flame is placed beneath one of its extremities. By this arrangement one can get a graduated temperature, beginning at the part of the plate above the gas-flame where it is hottest, and becoming gradually cooler toward the other end of the plate, which may be of a very low temperature. By dropping water upon the plate, beginning at the hottest point and proceeding toward the cooler end, it is easy to determine the point at which the water just boils; it is at a little below this point that the cover-slips are to be placed, bacteria side up, and allowed to remain about ten minutes, when the fixing will be complete. The same may be accomplished in a small copper drying-oven, which is regulated to remain at a temperature of from 95° to 98° C. In very particular work this plan is to be preferred to the process of passing the cover-slips through a flame, as the organisms are always subjected to the same degree of heat, and the distortions which sometimes occur from too great and irregular application of high temperatures may be eliminated. The fixing consists in drying or coagulating the gelatinous envelope surrounding the organisms, by which means they are caused to adhere to the surface of the cover-slip. It is sometimes desirable to fix the preparation without the use of heat, as in the case of pus or other exudates. In this event, after drying the thinly spread material carefully in the air, the cover-slip on which it is placed is

immersed in a mixture of equal parts of absolute alcohol and ether for about 15 minutes. At the end of this time it may be removed and stained. The advantage of this method is that there is less distortion and, as a rule, less precipitation (or, perhaps better, no charring) of extraneous matter. When fixed, staining is usually a simple matter. The majority of bacteria with which the beginner will have to deal stain readily with watery solutions of any of the basic aniline dyes, such, for instance, as fuchsin, methylene-blue, or gentian-violet.

To stain the fixed cover-slip preparation, it is taken by one of its edges between forceps, and a few drops of a watery solution of either of the dyes named are placed upon the film and allowed to remain twenty to thirty seconds. The slip is then carefully rinsed in water, and without drying is placed *bacteria down* upon a slide; the excess of water is taken up by covering it with blotting-paper and gently pressing upon it, after which the preparation is ready for examination.

Another plan sometimes used is to bring the slip upon the slide, *bacteria down*, without rinsing off the staining-fluid; the excess of fluid is removed with blotting-paper and the preparation is ready for examination with the microscope. This method is satisfactory and time-saving, but must always be practised with care. The staining-fluid should always be filtered before using, to rid it of insoluble particles which might be taken for bacteria. If upon examination the preparation proves of particular interest, so that it is desirable to preserve it, then it may be mounted permanently. The drop of immersion oil

is to be removed from the surface of the slip with blotting-paper, and the slip loosened, or rather floated, from the slide by allowing water to flow around its edges. It is then taken up with forceps, carefully deprived of the water adhering to it by means of blotting-paper, and allowed to dry. When dry it is mounted in xylol-Canada-balsam by placing a small drop of the balsam upon the surface of the film, and then inverting the slip upon a clean glass slide. It is sometimes desirable to have the balsam harden quickly, and a method that is commonly employed to induce this is as follows: the slide, held by one of its ends between the fingers, is warmed over a gas-flame until quite hot; a drop of balsam is then placed on the centre of it, and it is again warmed; the cover-slip is then placed in position, and when the balsam is evenly distributed the temperature is rapidly reduced by rubbing the bottom of the slide with a towel wet with cold water. Usually the preparation is firmly fixed after this treatment; a little practice is necessary, however, in order not to overheat and crack the slide. The method is applicable only to cover-slip preparations, and cannot be safely used with tissues.

Impression Cover-slip Preparations.—Impression preparations differ from ordinary cover-slip preparations in only one respect: they present an impression of the organisms as they were arranged in the colony from which the preparation is made. They are made by gently covering the colony with a thin, clean cover-slip, *lightly* pressing upon it, and, without moving the slip laterally, lifting it by one of its edges. The organisms adhere to the slip in the same relation to one another that they had in the colony. The subsequent

steps of drying, fixing, staining, and mounting are the same as those just given for ordinary cover-slip preparations.

By this method constancies in the arrangement and grouping of the individuals in a colony can often be made out. Some will always appear irregularly massed, others show growth in parallel bundles, while others, again, will be seen as long, twisted threads.

NOTE.—From a colony of *bacillus subtilis* make a cover-slip preparation in the ordinary way; now make an impression cover-slip preparation of another colony of the same organism. Compare the results.

ORDINARY STAINING-SOLUTIONS. — The solutions commonly employed in staining cover-slip preparations are, as has been stated, watery solutions of the basic aniline dyes—fuchsin, gentian-violet, and methylene-blue. These solutions may be made either by directly dissolving the dyes in substance in water until the proper degree of concentration has been reached, or by using concentrated watery or alcoholic solutions of the dyes which may be kept on hand as stock. The latter method is the one commonly practised.

The solutions of the colors which are in constant use in staining are prepared as follows:

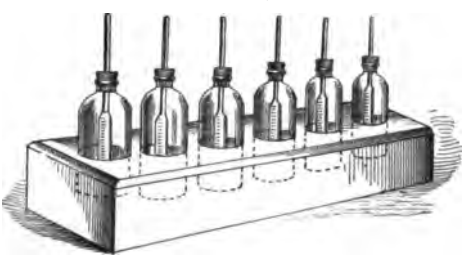
Prepare as stock, saturated alcoholic or watery solutions of fuchsin, gentian-violet, and methylene-blue. These solutions are best made by pouring into clean bottles enough of the dyes in substance to fill them to about one-fourth of their capacity. Each bottle should then be filled with alcohol or with water, tightly corked, well shaken, and allowed to stand for twenty-four hours.

If by then all the staining-material has been dissolved, more should be added, the bottle being again shaken and allowed to stand for another twenty-four hours; this must be repeated until a permanent sediment of undissolved coloring-matter is seen upon the bottom of the bottle. The bottles are then to be labelled "saturated alcoholic" or "watery" solution of fuchsin, gentian-violet, or methylene-blue, as the case may be. *The alcoholic solutions are not directly employed for staining-purposes.*

The solutions with which staining is accomplished are made from the stock solutions in the following way:

An ordinary test-tube of about 13 mm. diameter is three-fourths filled with distilled water and the concentrated alcoholic or watery solution of the dye added, little by little, until one can just see through the solution. It is then ready for use. Care must be taken that the color does not become too dense. The best results are obtained when this dilution is *just*

FIG. 35.



Rack of bottles for staining-solutions.

transparent as viewed through a layer about 12 to 14 mm. thick.

The above represent the staining-solutions in every-day use. They are kept in bottles supplied with stoppers and pipettes (Fig. 35), and when used are dropped upon the preparation to be stained.

For certain bacteria which stain only imperfectly with these simple solutions it is necessary to employ some agent that will increase the penetrating action of the dyes. Experience has taught us that this can be accomplished by the addition to the solutions of small quantities of alkaline substances, or by dissolving the staining-materials in strong watery solutions of either aniline oil or carbolic acid, instead of water—in other words, by employing special solvents and mordants with the stains.

Of the solutions thus prepared which may always be employed upon bacteria that show a tendency to stain imperfectly, there are three in common use—Löffler's alkaline methylene-blue solution; the Koch-Ehrlich aniline-water solution of either fuchsin, gentian-violet, or methylene-blue; and Ziehl's solution of fuchsin in carbolic acid. These solutions are as follows:

Löffler's alkaline methylene-blue solution:

Concentrated alcoholic solution of methylene-blue . .	30 c.c.
Caustic potash in 1 : 10,000 solution	100 c.c.

Koch-Ehrlich aniline-water solution. To about 100 c.c. of distilled water aniline oil is added drop by drop until the solution has an opaque appearance, the vessel containing the solution being thoroughly shaken after the addition of each drop. It is then filtered through moistened filter-paper until the filtrate is perfectly clear. To 100 c.c. of the clear filtrate add 10 c.c. of absolute alcohol and 11 c.c. of the concentrated alco-

holic solution of either fuchsin, methylene-blue, or gentian-violet, preferably fuchsin or gentian-violet.

Ziehl's carbol-fuchsin solution:

Distilled water	100 c.c.
Carbolic acid (crystallized)	5 grammes.
Alcohol	10 c.c.
Fuchsin in substance	1 gramme.

Or it may be prepared by adding to a 5 per cent. watery solution of carbolic acid the saturated alcoholic solution of fuchsin until a metallic lustre appears on the surface of the fluid.

The Koch-Ehrlich solution decomposes after a time, so that it is better to prepare it fresh in small quantities when needed than to employ old solutions. Solutions older than fourteen days should not be used.

The three solutions just given may be used for cover-glass preparations in the ordinary way.

In some manipulations it becomes necessary to stain the bacteria very intensely, so that they may retain their color when exposed to the action of decolorizing agents. These methods are usually employed when it is desirable to deprive surrounding objects or tissues of their color, in order that the stained bacteria may stand out in greater contrast. It is in these cases that the staining-solution with which the bacteria are being treated is to be warmed, and in some cases boiled, so as further to increase its penetrating action. When so treated, certain of the bacteria will retain their color, even when exposed to very strong decolorizers. The tubercle bacillus is distinguished from the great majority of other bacteria by the tenacity with which it retains the color when treated in this way; it is an

organism difficult to stain, but when once stained is equally difficult to rob of its color.

METHOD OF STAINING THE TUBERCLE BACILLUS. —Select from the sputum of a tuberculous subject one of the small, white, cheesy masses which it is seen to contain. Spread this upon a cover-slip, dry and fix it in the usual way. The slip is now to be taken by its edge with forceps and the film covered with a few drops of either the solution of Koch-Ehrlich or that of Ziehl. It is then held over a gas-flame, at first some distance away, gradually being brought nearer until the fluid begins to boil. After it has bubbled once or twice it is removed from the flame, the excess of stain washed away in a stream of water, then immersed in a 30 per cent. solution of nitric acid in water, and allowed to remain until all color has disappeared. This takes longer in some cases than in others. One can always determine if decolorization is complete by washing off the acid in a stream of water. If the preparation is still distinctly colored, it should be again immersed in the acid; if of only a very faint color, it may be dipped in alcohol, again washed in water, and stained with some contrast-color. If, for example, the tubercle bacilli have been stained with fuchsin, methylene-blue forms a good contrast-stain. In making the contrast-stain the steps in the process are exactly those followed in the ordinary staining of cover-slip preparations in general: the slip containing the stained tubercle bacilli is carefully rinsed in water, and a few drops of the methylene-blue solution placed upon it and allowed to remain for thirty or forty seconds, when it is again rinsed in water and examined microscopically. For the purpose of observ-

ing the difference in behavior of the tubercle bacilli and the other organisms present in the preparation toward this method of staining, it is well to examine the preparation microscopically before the contrast-stain is made; then give it the contrast-color, and again examine. It will be seen that before the contrast-color has been given to the preparation the tubercle bacilli are the only stained objects to be made out, and the preparation appears devoid of other organisms; but upon examining it after it has received the contrast-color a great many other organisms will appear; these take on the second color employed, while the tubercle bacilli retain their original color. Before decolorization all organisms in the preparation were of the same color, but during the application of the decolorizing solution all except the tubercle bacilli gave up their color. This micro-chemical characteristic, together with reactions to be described, serves to differentiate the tubercle bacillus from other organisms with which it might be confounded. A number of different methods have been suggested for the staining of tubercle bacilli, but the original method as employed by Koch is so satisfactory in its results that it is not advisable to substitute others for it. The above differs from the original Koch-Ehrlich method for the staining of tubercle bacilli in sputum only in the occasional employment of Ziehl's carbol-fuchsin solution and in the method of heating the preparation with the staining-fluid upon it.

As Nuttall has pointed out, however, the strong acid decolorizer used in this method can, with advantage, be replaced by much more dilute solutions, as a number of the bacilli are entirely decolorized by the too

energetic action of the strong acids. He recommends the following method of decolorization: after staining the slip or section in the usual way, pass it through three alcohols; it is then to be washed in a solution composed of

Water	150 c.c.
Alcohol	50 c.c.
Concentrated sulphuric acid	20 to 30 drops.

From this it is removed to water and carefully rinsed. The remaining steps in the process are the same as those given in the other methods.

GABBETT'S METHOD for the staining of tubercle bacilli recommends itself because of its simplicity and the rapidity with which it can be performed. By many it is considered the best method for routine employment. It consists in staining the cover-slips, prepared in the manner given, for from two to five minutes in a cold carbol-fuchsin solution, after which they are subjected to the action of Gabbett's methylene-blue sulphuric acid solution. This latter consists of

Sulphuric acid (strength 25 per cent.)	100 c.c.
Methylene-blue, in substance	1 to 2 grammes.

The cover-slips are then rinsed in water and are ready for examination. The tubercle bacilli will be stained red by the fuchsin, while all other bacteria, cell-nuclei, etc., will be tinted blue.

GRAM'S METHOD.—Another important differential method of staining which is very commonly employed is that recommended by Gram. In this method the objects are treated with an aniline-water solution of gentian-violet made after the formula of Koch-Ehrlich.

After remaining in this for twenty to thirty minutes they are immersed in a solution composed of

Iodine	1 gramme.
Potassium iodide	2 grammes.
Distilled water	300 c.c.

In this they remain for about five minutes; they are then transferred to alcohol and thoroughly rinsed. If still of a violet color, they are again treated with the iodine solution, followed by alcohol, and this is continued until no trace of violet is visible to the naked eye. They may then be examined, or a contrast-color of carmine or Bismarck-brown may be given them.

This method is particularly useful in demonstrating the capsule which is seen to surround some bacteria, especially *micrococcus lanceolatus* of pneumonia.

Glacial Acetic Acid Method.—Another method that may be employed for demonstrating the presence of the capsule surrounding certain organisms is to prepare the cover-slips in the ordinary way, then cover the layer of bacteria upon them with glacial acetic acid, which is instantly poured off (not washed off in water), and the aniline-water gentian-violet solution dropped upon them; this is allowed to remain three or four minutes, is poured off, and a few drops more are added, and lastly the slip is washed in a solution of sodium chloride. Usually this is of the strength of physiological salt-solution, viz., 0.6 to 0.7 per cent.; but at times it must be stronger, occasionally as concentrated as 1.5 to 2 per cent. The reason for this is that if the slips be washed in water, or in salt-solution that is too weak, the mucin capsule that has been coagulated by the acetic acid is redissolved

and rendered invisible. This does not occur when the salt-solution is of the proper strength—a point that can be determined only after a few trials with solutions of different strengths. (Welch.) A very clear, sharply cut picture usually follows this method of procedure.

Ribbert also recommends for the staining of capsulated bacteria the *momentary* immersion of the cover-slips in a saturated solution of dahlia in a mixture of 100 parts of water, 50 parts of alcohol, and 12½ parts of glacial acetic acid; after which the excess of color is removed by washing in water.

STAINING OF SPORES.—We have learned that one of the points by which spores may be recognized is their refusal to take up staining-substances when applied in the ordinary way. They may, however, be stained by special methods; of these, one that has given fairly satisfactory results in our hands is as follows: the cover-slip is to be prepared from the material containing the spores in the ordinary way, dried, and fixed. It is then to be held by its edge with forceps, and its surface covered with Löffler's alkaline methylene-blue solution. It is then held over the Bunsen flame until the fluid boils; it is then removed, and after a few seconds is heated again. This is continued for about one minute, after which it is washed in water and then decolorized in

Alcohol (80 per cent.)	98 c.c.
Nitric acid	2 c.c.

until all visible blue color has disappeared. It is then rinsed in water and dipped for from 3 to 5 seconds in

Saturated alcoholic solution of eosin	10 c.c.
Water	90 c.c.

after which it is again rinsed in water and finally mounted for examination. If the decolorization in the acid alcohol be not carried too far, the preparation will show the spores stained blue and the bodies of the cells to have taken on the rose color characteristic of eosin.

By another process the cover-slip is floated, bacteria down, upon the surface of freshly prepared Koch-Ehrlich solution of fuchsin contained in a watch-crystal. This is then held by its edge with forceps about 2 cm. above a very small flame of a Bunsen burner, care being taken that the flame touches only the centre of the bottom of the crystal. After a few seconds the crystal is gradually elevated until it is about 6 to 8 cm. above the flame; then it is slowly moved down to the flame again, and this up-and-down movement is continued until the staining-fluid begins to boil. As soon as a few bubbles have been given off it is held aside for a minute or two, when the heating is repeated. When the boiling begins the crystal is again held aside for a minute or two. The crystal is heated in this way five or six times. When the fluid has stood for about five minutes after the last boiling the preparation is transferred, without washing in water, to a second watch-crystal containing the following decolorizing solution:

Absolute alcohol	100 c.c.
Hydrochloric acid	3 c.c.

In this solution it is placed, bacteria up, and the vessel is tilted from side to side for about one minute. It is then removed, washed in water, and stained with the methylene-blue solution. The spores will be stained red and the body of the cells blue.

MOELLER'S METHOD FOR STAINING SPORES.—A

method that has recently been published by Moeller is designed to favor the penetration of the coloring-material through the spore-membrane by macerating the spores in a solution of chromic acid before staining them. It is as follows:

The cover-slips are prepared in the usual way, or the fixing may be accomplished with absolute alcohol instead of high temperatures. The preparation is held for two minutes in chloroform, washed in water, placed for from one-half to two minutes in a 5 per cent. solution of chromic acid, again washed in water, and stained with carbol-fuchsin. In the process of staining, the slip is taken by the corner with forceps, and carbol-fuchsin is dropped upon the side containing the spores. It is then held over a flame until it boils, and then held some distance above the flame for one minute. The staining-fluid is then poured off and the preparation is completely decolorized in 5 per cent. sulphuric acid, again washed in water, and finally stained for thirty seconds in the watery methylene-blue solution. The spores will be red, the body of the cells blue.

In this method the object of the preliminary exposure to chloroform is to dissolve any crystals of lecithin, cholesterin, or fat that may be in the preparation, and which when stained might cause confusion.

It must be remembered that there are conspicuous differences in the behavior of spores of different bacteria to staining-methods and of the spores of a single species in different stages of development. Some stain readily by either of the methods especially devised for this purpose, while others can hardly be stained at all, or only with the greatest difficulty, by any of the known processes; some stain readily when fully developed, but

with difficulty when only partly developed; others have this peculiarity reversed.

LOFFLER'S METHOD FOR STAINING FLAGELLA.—For the demonstration of the locomotive apparatus possessed by motile bacteria we are indebted to Löffler. By a special method of staining, in which the use of mordants played the essential part, he has shown that these organisms possess very delicate, hair-like appendages, by the lashing movements of which they propel themselves through the fluid in which they are growing. The method as given by Löffler is as follows:

It is essential that the bacteria be evenly and *not too numerously* distributed upon the cover-slip. The slips must therefore be perfectly clean. (See *Löffler's method* of cleaning cover-slips.) Five or six of the carefully cleansed cover-slips are to be placed in a line on a table, and on the centre of each slip a very small drop of tap-water is placed. From the culture to be examined a minute portion is transferred to the first slip and carefully mixed with the drop of water; from this mixture a small portion is transferred to the second, and from the second to the third slip, and so on, in this way insuring a dilution of the number of organisms present in the preparations. These slips are then dried and fixed in the ordinary way. They are next to be warmed in the following solution:

Tannic acid solution in water (20 acid, 80 water)	10 c.c.
Cold saturated solution of ferrous sulphate	5 c.c.
Saturated watery or alcoholic solution of fuchsin . . .	1 c.c.

This solution represents the mordant. A few drops of it are to be placed upon the film of bacteria on the cover-slip, which is then to be held over a flame until

the solution begins to steam. *It should not be boiled.* After steaming, the mordant is washed off in water and finally in alcohol. The bacteria are then to be stained in a saturated aniline-water-fuchsin solution.

When treated in this way different bacteria behave differently: the flagella of some stain readily in the above solutions; others require the addition of an alkali in varying quantities; while others stain best after the addition of acids. To meet these conditions an exact 1 per cent. solution of caustic soda in water must be prepared, and also a solution of sulphuric acid in water of such strength that one cubic centimetre will be exactly neutralized by one cubic centimetre of the alkaline solution.

For different bacteria which have been studied by this method Löffler recommends the one or the other of these solutions to be added to the mordant in the following proportions.

Of the acid solution:

For *spirillum choleræ Asiaticæ*,	½	to 1 drop of acid to 16 c.c. of mordant.			
For *spirillum rubrum*,	9	"	"	"	"
For *spirillum Mètchnikoffi*,	4	"	"	"	"
For *bacillus pyocyaneus*,	5	"	"	"	"

For *spirillum concentricum*, no addition of either acid or alkali.

Of the alkaline solution:

For *bacillus mesentericus vulgatus*,	4	drops of alkali to 16 c.c. of mordant.			
For *micrococcus agilis*,	20	"	"	"	"
For *bacillus typhosus*,	22	"	"	"	"
For *bacillus subtilis*,	28–30	"	"	"	"
For *bacillus œdematis maligni*,	36–37	"	"	"	"
For *bacillus anthracis symptomatici*,	35				

The drops used run 22 to the cubic centimetre.

For other organisms one must determine whether the results are better after the addition of acid or alkali,

and how much of either is required. In general, it may be said that bacteria which produce acids in the media in which they are growing require the addition of alkalies to the mordant, while those that produce alkalies require acids to be added. By following Löffler's directions the delicate, hair-like flagella on motile organisms may be rendered plainly visible.

There are several points and slight modifications in connection with this method that require to be emphasized in order to insure success: the culture to be employed should be young, not over 18-20 hours old; it should have developed for this time on fresh agar-agar at 37° to 38° C.; the mordant should not be perfectly fresh, as the best results are obtained from the use of old solutions that have stood exposed to the air and that have been filtered just before using; when placed on the cover-slip and held over the flame *never heat the mordant to the boiling-point; indeed, the best results are obtained when the preparation is held high above the flame and removed from it at the first evidence of vaporization, or, better still, a little before this point is reached.* We have derived no advantage from the addition of acids or alkalies to the mordant, as recommended by Löffler; but obtain, with a fair degree of regularity, satisfactory results through the use of the neutral mordant alone.[1]

BUNGE'S METHOD.—A useful modification of Löffler's method is that recommended by Bunge: prepare a saturated solution of tannin, and a solution of liquor ferri sesquichlor. of the strength of 1 : 20 of distilled water. To 3 parts of the tannin solution add 1 part of

[1] I am indebted to Dr. James Homer Wright, Thomas Scott Fellow in Hygiene, 1892-'93, University of Pennsylvania, for some of the suggestions in connection with the modification of this method.

the dilute iron solution. To 10 c.c. of such a mixture add 1 c.c. of concentrated watery solution of fuchsin. This mordant is not to be used fresh, but only after standing exposed to the air for several days (better for several weeks). After preparing the cover-slip with all precautions necessary to cleanliness the filtered mordant is allowed to act cold for about five minutes, after which it is slightly warmed; the slip is then washed in water, dried, and faintly stained with carbol-fuchsin. No addition of acid or alkali to the mordant is necessary.

[illegible] of Van Ermengem.—Another method of demonstrating the presence of flagella is that [illegible] Van Ermengem. It is somewhat more [illegible] than either of the preceding methods. The [illegible] as follows:

[illegible] a cover-slip place [illegible] physiological salt- [illegible] culture of [illegible] The suspension of the [illegible] should be very dilute in order to allow the isolation of single cells on the slip and also to avoid the occurrence of excessive precipitation. The slips are then to be dried in the air and fixed over a gas flame in the usual manner.

The mordant used consists of:

[illegible]	1 part.
[illegible]	2 parts.

To the mordant 1 or 5 drops of glacial acetic acid may be added, but experience has shown this to be hardly necessary. Place a drop or two of the mordant on the cover-slip to be stained, and allow it to act for one-half

hour at room-temperature, or for five minutes at 50° to 60° C. Wash carefully in water and in alcohol, and then immerse for a few seconds in the "sensitizing bath," viz., a 0.25–0.5 per cent. solution of silver nitrate. Without washing, bring the slip into a watch-crystalful of the "reducing and reinforcing bath," viz.:

Gallic acid	5 grammes.
Tannin	3 "
Fused potassium acetate	10 "
Distilled water	350 "

After a few seconds pass the slip back into a watch-crystal containing the dilute silver bath (0.25–0.5 per cent. solution of silver nitrate in water) and keep it in constant motion until the solution begins to take on a brown or blackish color. Wash in water *thoroughly;* dry with blotting-paper, and mount in balsam.

BOWHILL'S METHOD.—From a fresh culture on agar-agar make a suspension in sterilized distilled water in a test-tube; allow this to stand for five minutes; from the supernatant fluid prepare cover-slips, care being taken that the slips are clean and that too much heat is not applied in fixing; float the cover-slip, bacteria down, upon some of the following mixture in a watch-glass:

Saturated alcoholic solution of orcein (about 10 days old)	15 c.c.
20 per cent. solution of tannic acid in water (acid dissolved by heating)	10 c.c.
Distilled water	30 c.c.

Gently warm the fluid and allow the preparation to remain in it for 10–15 minutes; then rinse in water, and finally mount and examine in water or dry and mount in xylol balsam.[1]

[1] Bowhill: Hygienische Rundschau, 1898, No. 3, p. 105.

STAINING IN GENERAL.

The physics of staining and decolorization is hardly a subject to be discussed at length in a book of this character; but, as Kühne has pointed out, it may be briefly said that solutions which favor the production of diffusion-currents facilitate intensity of staining, and by a similar process increase the energy of decolorizing-agents. For example, tissues which are transferred from water into watery solutions of the coloring-matters are less intensely stained and more easily decolorized than when transferred from alcohol into watery staining-fluids; for the same reason tissues stained in watery solutions of the dyes do not become decolorized so readily when placed in water as when placed in alcohol.

The diffusion of staining-solutions in the protoplasm of dried bacteria, as found upon cover-slip preparations, is much greater and more rapid than when the same bacteria are located in the interstices of tissues. These differences are not in the bacteria themselves, but in the obstruction to diffusion offered by the tissues in which they are located. The result of absence of diffusion may easily be illustrated:

Prepare a cover-slip preparation, dry it carefully, fix it, and, without allowing water to get on it from any source, attempt to stain it with a solution of the dyes in *absolute alcohol*, washing it subsequently with absolute alcohol; the result is negative. The absolute alcohol does not possess the property of diffusing into the dried tissues, and hence, as has been stated, alcoholic solutions of the staining-dyes should not be

employed. The staining-dyes should always be watery.[1]

DECOLORIZING-SOLUTIONS.—As regards the employment of decolorizing-agents, it must always be borne in mind that objects which are easily stained are also easily decolorized, and those that can be caused to take up the staining-material only with difficulty are also very difficult to rob of their color. The most common decolorizer in use is probably alcohol—not absolute alcohol, but alcohol containing more or less of water. Water alone has this property, but in a much less degree than dilute alcohol. On the other hand, a much more energetic decolorization than that possessed by either alone can be obtained by alternate exposures to alcohol and water. More energetic in their decolorizing action than either water or alcohol are solutions of the acids. They appear, particularly when they are alcoholic solutions, to diffuse rapidly into tissues and bacteria and very quickly extract the staining-materials which have been deposited there. For this reason these solutions should be employed with much care.

Very dilute acetic acid robs tissues and bacteria of their stain with remarkable activity; still more energetic are solutions of the mineral acids, and particularly, as has been said, when this action is accompanied by the decolorizing-properties of alcohol.

The acid solutions commonly employed are:

Acetic acid in from 0.1 to 5 per cent. watery solution.

[1] In the beginning of this chapter it was stated that the saturated alcoholic solutions of the dyes do not serve as stains for bacteria. It must be remembered that this holds only when absolute alcohol and perfectly dry coloring-matters have been used. If but a small proportion of water is present, the bacteria may be stained with these solutions, though the results are, as a rule, unsatisfactory.

Nitric acid in from 20 to 30 per cent. watery solution.

Hydrochloric acid in from 1 to 3 per cent. solution in alcohol.

STAINING OF BACTERIA IN TISSUES.

In staining tissues for the purpose of demonstrating the bacteria which they may contain a number of points must be borne in mind: the conditions which favor the diffusion of the staining-fluids into the bacteria are now not so favorable to rapid staining as they were when the bacteria alone were present upon cover-slips; the staining of tissues, therefore, requires a longer exposure to the dyes than does that of cover-slips. In tissues, too, there are other substances beside the bacteria which become stained, and these, unless robbed in whole or in part of their color, may so mask the stained bacteria as to render them difficult, if not impossible, of detection. Tissues must, therefore, always be subjected to some degree of decolorization, and this must be accomplished without depriving the bacteria of their color.

The details of the method of decolorization will be described in the section on the technique of staining.

Another point to be remembered in staining tissues is that they cannot be heated and retain their structure in the same way that one heats cover-slips. The best results are not obtained in efforts to hasten the staining by subjection to high temperatures, but rather by longer exposures to lower temperatures.

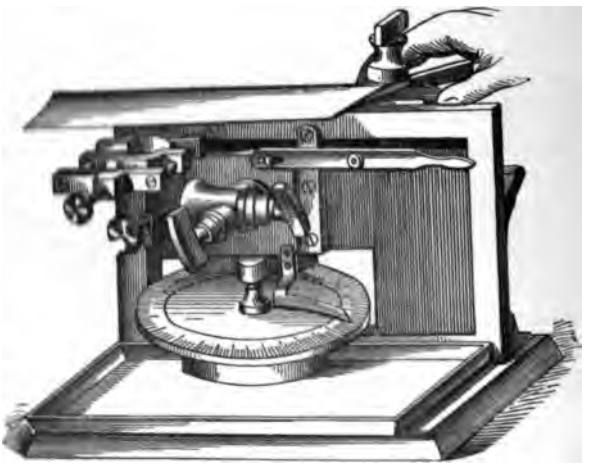
HARDENING THE TISSUES.—The bits of tissue—not larger than one cubic centimetre—are to be placed, as fresh as possible, in absolute alcohol. They should rest upon a pad of cotton or filter-paper in the bottle con-

taining the alcohol, in order that they may be elevated and surrounded by the portion of the alcohol which is specifically the lightest, and consequently contains least water. The alcohol abstracts water from the tissue, and as the dehydration proceeds the tissue becomes accordingly more and more dense. When of about the consistency of fresh solid rubber, or preferably not quite so dense, it is ready to cut. A small portion, about half a cubic centimetre, should be cemented to a bit of cork with ordinary mucilage, and allowed to remain in the open air for a minute or two for the mucilage to harden. Alcohol should be dropped upon it occasionally to prevent drying of the tissue. When the mucilage is hard the cork with the piece of tissue upon it may be left in alcohol over night, and on the following day the sections may be cut.

SECTION-CUTTING.—This is accomplished by the use of an instrument known as a microtome. In Fig. 36 is seen the form now commonly employed. It is known as Schanze's microtome. It is an apparatus provided with a clamp for holding the cork upon which the tissue is cemented, and also a sliding clamp which carries a knife. The tissue is clamped horizontally, and the knife is caused to slide across its upper surface, also in a horizontal plane. Beneath the clamp for holding the tissue is a milled disk, by means of which a screw is caused to revolve, and in revolving raises or lowers the clamp holding the tissue, so that the tissue may be brought closer to or farther from the plane in which the knife slides. By this arrangement sections of any desired thickness can be cut by turning the milled disk with the one hand and causing the knife to traverse the tissue with the other. The tissue and the knife-blade should be kept wet

with alcohol, so that the sections may float upon the blade of the knife, from which they can easily be removed, without tearing, with a curved needle or a

FIG. 36.



Schanze's microtome.

camel's-hair brush. As the sections are cut they are placed in a dish containing alcohol.

There are some tissues which, by reason of their histological structure, do not become sufficiently dense when exposed to alcohol to permit of their being cut in the above way. It becomes necessary to render them more solid by filling their interstices with some substance that neither interferes with their structure nor prevents their being cut into sections. They must be "imbedded," as this process is called.

Imbedding in celloidin. Most convenient for this purpose is celloidin, a body somewhat similar to collodion, soluble in a mixture of equal parts of alcohol and ether, as well as in absolute alcohol.

After hardening in alcohol the tissue to be imbedded is placed in a mixture of equal parts of absolute alcohol and ether and left there for twenty-four hours. It is then transferred to celloidin. Two solutions of celloidin are to be employed: the one a thin solution in a mixture of equal parts of absolute alcohol and ether, the other a thick solution in the same solvent. Into the thin solution, which should be of about the consistence of very thin syrup, the tissue is placed from the absolute alcohol and ether, and allowed to remain for twenty-four hours. It is then placed in the thick solution for about a day. When removed it should be placed immediately upon a bit of cork or a block of wood. The adherent celloidin acts as a cement; as it hardens rapidly the tissue is soon fast to the cork. The section is then left in 60 per cent. alcohol for twenty-four hours to complete the solidification of the celloidin, after which sections may be cut in the way just described for tissues not so treated.

Imbedding in paraffin. After bits of the tissue not larger than a cubic centimetre have been hardened in the usual way they are placed in fresh *absolute* alcohol for twenty-four hours to complete the process. From this they are transferred to pure turpentine, and kept in a warm oven at a temperature not exceeding 35° to 38° C. Here they remain until thoroughly saturated with the turpentine, as is recognized by the transparent appearance they assume. From this they are placed in paraffin that melts at 53° C., and allowed to remain in this for three or four hours. They are then transferred to a small paper or metal mould, or a pill-box, and melted paraffin is poured over them. When the paraffin has become solid the mould or box is removed

from around it, the excess of paraffin trimmed from about the imbedded tissue, and the latter is ready for cutting.

When the sections are cut they are freed from paraffin by exposing them to turpentine; the latter is removed by washing in alcohol and the sections can then be stained in the ordinary way. In cutting sections from tissues that have been imbedded in paraffin the long axis of the knife should be at nearly right angles to the direction in which the knife travels. For bacteriological purposes the method of imbedding in paraffin does not, as a rule, give such good results as when the celloidin method is employed. In this work, therefore, the latter is usually preferred.

STAINING THE SECTIONS.—The sections when cut may be stained in a variety of ways. The ordinary watery solutions of the three common basic aniline dyes—fuchsin, gentian-violet, and methylene-blue—or, what is better, the alkaline methylene-blue solution of Löffler, may be employed for general use.

Some of the acid aniline dyes, as well as some of the vegetable coloring-matters, are essentially nuclear stains, and are not applicable to the staining of bacteria.

Into a watch-glass containing either of the staining-solutions mentioned the sections are to be placed after having been in water for about one minute. They remain in the staining-solutions for from five to eight minutes. They are then removed, rinsed in water, and partly decolorized in 0.1 per cent. solution of acetic acid for only a few seconds; again washed in water, then in absolute alcohol for a few seconds, and from this again into absolute alcohol for the same time, and finally into cedar-oil or xylol. Here they remain for from one-half

to three-fourths of a minute. They are now to be evenly spread upon a spatula, which is held under them in the fluid, and, without draining off the fluid, are transferred to a clean glass slide. This must be done carefully to avoid tearing. The easiest way to do this is to hold the spatula on which the section floats in one hand, with its point just touching the surface of the glass slide, and then with a needle gently pull the section upon the slide. The fluid comes with it, and the floating section may be easily spread out into a flat surface. The excess of fluid is taken up with blotting-paper, after which a drop of xylol-balsam is placed upon the centre of the section, which is then covered with a thin, clean cover-slip. It is now ready for examination.

Each step in the above process has its definite object. The sections are placed in water before staining in order that the diffusion of the staining-solution into the tissues may be diminished; otherwise our efforts at rendering the bacteria more conspicuous by decolorizing the tissues in which they are located would rob the bacteria of their color as well.

The acetic acid and also the alcohol are decolorizers. They are employed to reduce the excess of stain in the tissues, though they also have some decolorizing action upon the bacteria. The cedar-oil and xylol are bodies which mix on the one hand with alcohol, and on the other with balsam. They are known as "clearing-fluids," and not only serve to differentiate the component parts of the tissue, but fill up the gap that would otherwise be left in the process, for a section cannot be mounted in balsam directly from alcohol; the two bodies do not mix perfectly. A number of clearing-

agents are in general use; in fact, almost all the essential oils come under this head. There is one—oil of cloves—which is very commonly used in histological work; but it must not be employed for tissues containing bacteria. It not only extracts too much color from the bacteria, but causes them to fade after the sections have been mounted for a time.

When the section thus stained and mounted is examined microscopically it may be found that the tissues still possess so much color that the bacteria are not visible, in which case they have not been decolorized sufficiently; or, on the other hand, both bacteria and tissues may have parted with their stains—then decolorization has been carried too far. In either case the fault must be remedied in the manipulation of the next section to be mounted.

In short, the steps in the process of staining sections in general are these:

a. From alcohol into distilled water for one minute.

b. Into the staining-fluid for from five to eight minutes.

c. Into water from three to five minutes.

d. Into 0.1 per cent. acetic acid for about one-half minute.

e. Into absolute alcohol for a few seconds.

f. Into absolute alcohol again for a few seconds.

g. Xylol for about one-half minute.

h. Removal with spatula or section-lifter to slide.

i. Removal of excess of xylol.

j. Mounting in xylol-balsam.

The section must be lifted from one vessel to the other by means of either a curved needle or a glass rod drawn out to a fine end and bent in the form of a curved needle.

By the above process of staining, which can be practised as a routine method for most bacteria in tissues, the nuclei of the tissue-cells, as well as the bacteria, will be more or less deeply stained.

Special Methods of Staining Bacteria in Tissues.—For purposes of contrast-stains it sometimes becomes necessary to decolorize completely, or nearly completely, the tissues and leave the bacteria unaltered in color. For this purpose special methods depending on the staining-peculiarities of the bacteria under consideration have been devised.

Gram's method with tissues. One of the most commonly employed differential stains is that of Gram. In general, it is practised in the way given for its employment on cover-slip preparations, with some slight modifications.

In this method the sections are transferred from water into a solution of aniline-water gentian-violet, as prepared by the Koch-Ehrlich formula, but which has been diluted with about one-third its volume of water. In this the sections remain for about ten minutes, preferably in a warm place, at a temperature of about 40° C. They should never, under any conditions, be boiled. From this they are washed alternately in the iodine solution and alcohol, occasionally renewing the stained with clean alcohol, until all color has been extracted from them. They are then brought for one minute into a dilute watery solution of eosin or safranin, or Bismarck-brown, again washed for a few seconds in alcohol, and finally for one-fourth minute in absolute alcohol. From this they are transferred to xylol for a half-minute. The remaining steps in the process are the same as those given in the general method. In

some cases better results are obtained by reversing the steps in the process and staining the bacteria last, for then the repeated decolorizing action of the alcohol on the bacteria is diminished; thus, place the sections from alcohol into eosin, safranin, or Bismarck-brown for a few minutes, then wash out in 50 per cent. alcohol, then for from three to five minutes in the dilute aniline-water gentian-violet solution, then into the iodine bath; after three minutes wash out in alcohol, and, finally, for one-fourth minute in absolute alcohol, and then into the xylol, from which they may be mounted. Some of the organisms which may be stained by this method are *micrococcus tetragenus*, *bacillus diphtheriæ*, *bacillus anthracis*, and *staphylococcus pyogenes aureus*. It cannot be successfully employed with the bacillus of typhoid fever, nor with tissues containing *micrococcus gonorrhœæ*.

Staining with dahlia and decolorizing with sodium carbonate solution. Another method that is not very commonly employed, though the results obtained by its use are in many cases very satisfactory, is to stain the tissues in a strong watery solution of dahlia (about one-fourth saturated) for from ten to fifteen minutes; from this they are transferred to a 2 per cent. solution of sodium or potassium carbonate, and from this into alcohol, alternating from the one to the other until the sections are almost colorless. After the alcohol they are rinsed in water and then put into a dilute watery solution of either eosin, Bismarck-brown, or safranin for one minute, then washed out in alcohol, finally in absolute alcohol, and then in xylol, from which they may be mounted in the manner given.

Especially brilliant results are obtained when tissues containing anthrax bacilli are stained by this process;

the bacilli will be of a deep-blue color, while the surrounding tissues will be of the color used as contrast.

Kühne's carbolic methylene-blue method. Stain the sections in the following solution for from one-half to one hour:

Methylene-blue, in substance	1.5 grammes.
Absolute alcohol	10 c.c.

Rub up thoroughly in a mortar, and when the blue is completely dissolved add gradually 100 c.c. of a 5 per cent. solution of carbolic acid. (This solution decomposes after a short time; it should be made fresh when needed.) From this the sections are washed in water, then in 1.5 to 2 per cent. hydrochloric acid in water, after which they are transferred to a solution of lithium carbonate of the strength of six to eight drops of a concentrated watery solution of the salt to ten drops of water, and from this they are again thoroughly washed in water, then in absolute alcohol containing enough methylene-blue in substance to give it a tolerably dense color, then for a few minutes in aniline oil to which a little methylene-blue in substance has been added, then completely rinsed out in pure aniline oil; from this they are passed into thymol or oil of turpentine for two minutes, and then into xylol, from which they are mounted in xylol-balsam. The advantages of this method are that it is generally applicable, and by its use the bacteria are not robbed of their color, whereas the tissues are sufficiently decolorized to render the bacteria visible and admit of the use of contrast-stains.

Weigert's modification of Gram's method for sections. Stain the sections in the Koch-Ehrlich aniline-water gentian-violet solution for five or six minutes; wash

in water or physiological salt-solution (0.6 to 0.7 per cent. solution of sodium chloride in distilled water); transfer them with the section-lifter to the slide; take up the excess of fluid by gently pressing upon the flat section with blotting-paper; treat the section with the iodine solution used by Gram; take up the excess of the solution with blotting-paper; cover the section with aniline oil—this not only differentiates the component parts of the section, but dehydrates as well; wash out the aniline oil with xylol, and mount in the usual way in xylol-balsam. Or, decolorization with iodine may be omitted, and the sections, after staining in the aniline-water gentian-violet for five or six minutes—or longer, if necessary—are transferred to the slide without being washed in water or salt-solution (or, if so, only very slightly and rapidly), dried as completely as possible with filter-paper, and then decolorized with a mixture of aniline oil (one part) and xylol (two parts). This is the delicate part of the process, and can be watched under a low power of the microscope. When decolorization is sufficient (repeated applications of the aniline oil and xylol mixture are generally necessary) pure xylol replaces the mixture, and the specimen is finally mounted in xylol-balsam. Unless all the aniline oil is replaced by the xylol the specimen will not keep well. In this process the aniline oil is really the decolorizer, and has the valuable property of absorbing a certain amount of water, so that dehydration with alcohol is avoided. This method, while it stains certain bacteria in tissues very satisfactorily, is nevertheless designed especially for the staining of fibrin. Fibrin and hyaline material will be stained deep blue; bacteria a dark violet.

STAINING TUBERCLE BACILLI IN TISSUES.—As in the case of cover-slips, only those methods most commonly employed will be given.

The method of Ehrlich. Stain the sections in aniline-water fuchsin or gentian-violet for twenty-four hours; decolorize in 20 per cent. nitric acid for a few seconds only—the color need not be entirely extracted; then in 70 per cent. alcohol until no more color can be extracted by the alcohol; stain as contrast-color in dilute watery methylene-blue, malachite-green, or Bismarck-brown solution; wash out in 90 per cent. alcohol, then in absolute alcohol for a few seconds; clear up in xylol and mount in xylol-balsam.

Method of Ziehl-Neelsen. Stain the sections in warmed carbol-fuchsin solution for one hour; temperature to be about 45° to 50° C. Decolorize for a few seconds in 5 per cent. sulphuric acid, then in 70 per cent. alcohol, and from this on as by the Ehrlich method.

Dry method. For tubercle bacilli, as for many other organisms in tissues, the following method may be employed if only the presence of organisms is to be detected and the histological condition of the tissues is a matter of no consequence: bring the sections from water upon a slide or cover-slip, dry, fix, and stain by the methods for cover-slip preparations.

Gray's method. The method employed by Gray at the Army Medical Museum, Washington, D. C., a description of which is given by Borden, is as follows: the tissue to be stained should be hardened preferably in alcohol, in pieces not exceeding 1.5 by 1.5 by 1 cm. in size, though tissues hardened by any other of the regular methods can be stained. Alcohol is to be preferred, however, as after its use the bacilli stain

more quickly and brilliantly than when one of the other hardening fluids—Müller's, for instance—is employed.

After the tissue has been hardened it is imbedded in *paraffin* and cut in the usual manner. The sections are then cemented to the slides with a filtered ½ per cent. solution of gold-label gelatin, to which is added chloral hydrate in the proportion of 1 per cent., as a preservative. Several drops of this are placed on each slide, a section laid on top, and the slides placed in a warming-oven kept at a temperature slightly *below* the melting-point of the paraffin. In about five minutes all wrinkles will have been taken out of the sections, which will lie perfectly flat and smooth on the surface of the gelatin solution. The slides are then removed from the oven and the surplus fluid poured from them, thus bringing the sections in contact with their surface, after which they are set aside in a place protected from dust, to remain until the sections are firmly cemented to them by the drying of the gelatin solution. The drying may be hastened by keeping the slides in an oven below the melting-point of the paraffin; but it is best to set the slides aside until the next day, when the sections will be found to be perfectly cemented to them. The paraffin is then removed from the sections by turpentine, the turpentine by absolute alcohol, the absolute alcohol by 50 per cent. alcohol, and this by water, after which the slides are placed for five minutes in a 5 per cent. aqueous solution of potassium bichromate. This renders the gelatin insoluble and prevents the sections leaving the slides during their necessarily more or less prolonged immersion in the fuchsin stain. The potassium bichromate is washed out with water, and the slides

are then placed in a fuchsin stain, which is prepared as follows:

Fuchsin	1.5	grammes.
Absolute alcohol	14	c.c.
Carbolic acid crystals (pure)	6	grammes.
Water	100	c.c.

Dissolve the fuchsin in the alcohol and the carbolic acid in the water. Mix the two solutions and let stand for twelve hours, with occasional shaking or stirring; then filter.

The length of time that the slide remains in this solution varies with circumstances. Tubercle bacilli stain very quickly; in tissues properly hardened in alcohol five minutes are generally sufficient to stain them deeply.

Prolonged immersion in the fuchsin does no harm and insures certainty of results. After a section has been in the stain a sufficient length of time it, with the slide to which it is cemented, is washed in water until the surplus stain is removed; it is then subjected to the action of a combined decolorizer and contrast-stain made as follows:

Methyl-blue	2.25	grammes
Absolute alcohol	30	c.c.
Sulphuric acid	12	"
Water (distilled)	100	"

Dissolve the methyl-blue in the alcohol, add the acid to the water, mix the two solutions, and let stand, with occasional shaking, for twelve hours; then filter.

This solution is allowed to act upon the tissue for a few seconds, and as soon as the blue color predominates over the red, as seen by transmitted light, the section is immediately washed in water. Generally the red color

reappears, and the section must be again subjected to the action of the blue solution and again washed in water. This must be repeated until the blue almost, if not quite completely and permanently, replaces the red stain. This is the most important part of the process, and entirely satisfactory results are only obtained after some practice. The tendency is usually not to replace sufficiently the fuchsin with the methyl-blue, and in consequence the red color of the bacilli is masked by the red of the surrounding tissues. Unless all acid is thoroughly removed by the final washing in water the stain is not permanent. The section is then completely dehydrated with absolute alcohol, after taking up the excess of water on the slide with blotting-paper. The alcohol is followed by turpentine, and the process is completed by mounting in xylol-balsam.

In case it is desired to stain sections cut by the freezing method, they are placed upon a slide on which a few drops of the gelatin fixative have been placed, and after about five minutes, during which the fixative will have penetrated the section, the surplus is poured from beneath the section. The slides are then set aside for the gelatin to harden by drying, and after drying they are placed in bichromate fluid to render the gelatin insoluble. They are then manipulated in exactly the same manner as the sections cut by the paraffin method.

This method gives equally as good results with tissues containing the lepra bacillus as with those containing tubercle bacilli.

CHAPTER XI.

Systematic study of an organism—Points to be considered in identifying an organism as a definite species.

After isolating an organism by the plate method considerable work is necessary in order to establish its identity as a definite species. It must possess certain morphological and cultural peculiarities, which must be constant under constant conditions. Its form at different stages must always be the same. Its ability or inability to produce spores must not vary under proper conditions. Its growth upon different media under constant conditions of temperature and reaction must always present the same outward appearances. The changes brought about by it in the reaction of the media in which it is growing must follow a fixed rule. Its power to produce liquefaction of the gelatin, or to grow upon it without bringing about this change, must always be the same. Its motility or non-motility, and, if motile, the approximate number and position of its organs of locomotion, must be determined. Its production of certain chemical products must be established by chemical analysis. Its behavior toward oxygen—*i. e.*, Does it require this gas for its growth? Is this gas an indifferent factor? or, By its presence are the life-processes of the organism checked?—must be decided. Its behavior under varying conditions of temperature and under the influence of different chemical bodies, as well as its growth in media of different reactions, is

to be studied. The property of producing fermentation with the liberation of gases, and the character and quantitative relations of these gases, must be ascertained. If it produces pigment, what are the conditions favorable and unfavorable to this function. And, lastly, we must consider its behavior when introduced into the bodies of animals used for experimental work—*i. e.*, Is it a disease-producing organism, or does it belong to the group of innocent saprophytes?

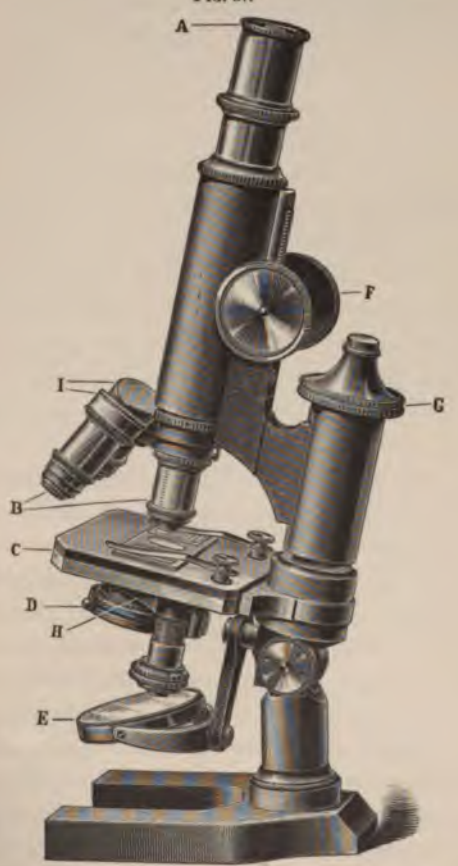
We have learned the methods of obtaining colonies, and have acquainted ourselves with some of the peculiarities by which they are distinguished from one another. The next important steps are to determine the morphology of the individuals composing these colonies, as well as their relation to each other in the colony. These points are decided by microscopic examination of bits of the colony, which are transferred to thin glass cover-slips, upon which they are dried, stained, and mounted. Cover-slips for this purpose are prepared in two ways: either by taking up a bit of the colony on a platinum needle, smearing it upon a cover-slip, staining it, and examining it—by which only the morphology of the individual bacteria can be made out—or by the method of "impression cover-slip preparations," by which not only the morphology, but also the relation of the organisms in the colony to one another can be determined. The details of these methods will be found in the chapter on the Methods of Staining.

MICROSCOPIC EXAMINATION OF PREPARATIONS.

THE DIFFERENT PARTS OF THE MICROSCOPE.—Before describing the method of examining preparations microscopically, a few definitions of the terms

used in referring to the microscope may not be out of place. (The different parts of the microscope referred to below are indicated by letters in Fig. 37.)

FIG. 37.



The *ocular* or *eye-piece* (A) is the lens at which the eye is placed when looking through the instrument. It

serves to magnify the image projected through the objective.

The *objective* (B) is the lens which is at the distal end of the barrel of the instrument, and which serves to magnify the object to be examined.

The *stage* (C) is the shelf or platform of the microscope on which the object to be examined rests.

The *diaphragms* are the perforated stops that fit in the centre of the stage. They vary in size, so that different amounts of light may be admitted to the object by using diaphragms with larger or smaller openings.

The "*iris*" *diaphragm* (D) opens and closes like the iris of the eye. It is so arranged that its opening for admission of light can be increased or diminished by moving a small lever in one or another direction.

The *reflector* (E) is the mirror placed beneath the stage, which serves to illuminate the object to be examined.

The *coarse adjustment* (F) is the rack-and-pinion arrangement by which the barrel of the microscope can be quickly raised or lowered.

The *fine adjustment* (G) serves to raise and lower the barrel of the instrument very slowly and gradually.

For the microscopic study of bacteria it is essential that the microscope be provided with an oil-immersion system and a sub-stage condensing apparatus.

The *oil-immersion* or *homogeneous system* consists of an objective so constructed that it can only be used when the transparent media through which the light passes in entering it are all of the same index of refraction—*i. e.*, are *homogeneous*. This is accomplished by interposing between the face of the lens and the cover-slip covering the object to be examined a body which refracts the

light in the same way as do the glass slide, the cover-slip, and the glass of which the objective is made. For this purpose a drop of oil of the same index of refraction as the glass is placed upon the face of the lens, and the examinations are made through this oil. There is thus no loss of light from deflection, as is the case in the dry system.

The *sub-stage condensing apparatus* (H) is a system of lenses situated beneath the central opening of the stage. They serve to condense the light passing from the reflector to the object in such a way that it is focussed upon the object, thus furnishing the greatest amount of illumination. Between the condenser and reflector is placed the "iris" diaphragm, the aperture of which can be regulated, as circumstances require, to permit of either a very small or a very large amount of light passing to the object.

The *nose-piece* (I) consists of a collar, or group of collars joined together (two or more), that is attached to the distal end of the tube of the microscope. It enables one to attach several objectives to the instrument in such a way that by simply rotating the nose-piece the various lenses of different power may be conveniently used in succession.

MICROSCOPIC EXAMINATION OF COVER-SLIPS.—The stained cover-slip is to be examined with the oil-immersion objective, and with the diaphragm of the sub-stage condensing apparatus open to its full extent. The object gained by allowing the light to enter in such a large volume is that the contrast produced by the colored bacteria in the brightly illuminated field is much more conspicuous than when a smaller amount of light is thrown upon them. This is true not only for stained bacteria on

cover-slips, but likewise for their differentiation from surrounding objects when they are located in tissues. With *unstained* bacteria and tissues, on the contrary, the structure can best be made out by reducing the bundle of light-rays to the smallest amount compatible with distinct vision, and in this way favoring, *not color-contrast*, but contrasts which appear as *lights and shadows*, due to the differences in permeability to light of the various parts of the material under examination.

Steps in Examining Stained Preparations with the Oil-immersion System.—Place upon the centre of the cover-slip which covers the preparation a small drop of immersion oil. Place the slide upon the centre of the stage of the microscope. With the coarse adjustment lower the oil-immersion objective until it *just touches* the drop of oil. Open the illuminating apparatus to its full extent. Then, with the eye to the ocular and the hand on the fine adjustment, turn the adjusting-screw toward *the right* until the field becomes somewhat colored in appearance. When this is seen proceed more slowly in the same direction, and, after one or two turns, the object will be in focus. *Do not remove the eye from the instrument until this has been accomplished.*

Then, with one hand upon the fine adjustment and the thumb and index finger of the other hand holding the slide lightly by its end, it may be moved about under the objective. At the same time the screw of the fine adjustment must be turned back and forth, so that the different planes of the preparation may be brought into focus one after the other. In this way the whole section or preparation may be inspected. When the examination is finished raise the objective from the

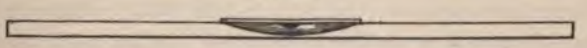
preparation by turning the screw of the *coarse* adjustment *toward you*. Remove the preparation from the stage, and, with a fine silk cloth or handkerchief, *wipe very gently and carefully* the oil from the face of the lens. The lens is then unscrewed from the microscope and placed in the case intended for its reception.

During work, of course, the lens need not be cleaned and put away after each examination; but when the work for the day is over an immersion lens must always be protected in this way. Under no circumstances should it be allowed to remain in the immersion oil or exposed to dust for any length of time.

EXAMINATION OF UNSTAINED PREPARATIONS.—"*Hanging drops.*" It frequently becomes necessary to examine bacteria in the unstained condition. The circumstances calling for this arise while studying the multiplication of cells, the germination of spores, the formation of spores, and the absence or presence of motility.

In this method the organisms to be studied are suspended in a drop of physiological salt-solution or bouillon in the centre of a cover-slip. This is then placed, drop down, upon a slide in the centre of which a hollow or depression is ground (Fig. 38). The slip is held in

FIG. 38.



Longitudinal section of hollow-ground glass slide for observing bacteria in hanging drops.

position by a thin layer of vaselin, which may be painted around the margins of the depression. This not only prevents the slip changing its position during examination, but also prevents drying by evaporation

if the preparation is to be observed for any length of time. This is known as the "hanging-drop" method of examination or cultivation. It is indispensable for the purposes mentioned, and at the same time requires considerable care in its manipulation. The fluid is so transparent that the cover-slip is often broken by the objective being brought down upon the preparation before one is aware that the focal distance has been reached. This may be avoided by grasping the slide with the left hand and moving it back and forth under the objective as it is moved toward the object. As soon as the *least pressure* is felt upon the slide the objective must be raised, otherwise the cover-slip will be broken and the lens may be rendered worthless.

A safer plan is to bring the *edge* of the drop into the centre of the field with one of the higher power *dry* lenses. When this is accomplished substitute the immersion for the dry system, when the edge of the drop is readily detected with the higher power lens somewhere near the centre of the field.

In examining bacteria by this method there is a possibility of error that must be guarded against. All microscopic insoluble particles in suspension in fluids possess a peculiar tremor or vibratory motion, the so-called "Brownian motion." This is very apt to give the impression that the organisms under examination are motile, when in truth they are not so, their movement in the fluid being only this molecular tremor.

The difference between the motion of bodies undergoing this molecular tremor and that possessed by certain living bacteria is that the former particles never move from their place in the field, while living bacteria alter their position in relation to the surround-

ing organisms, and may dart from one position in the field to another. In some cases the true movement of bacteria is very slow and undulating, while in others it is rapid and darting. The molecular tremor may be seen with non-motile and with dead organisms.

NOTE.—Prepare three hanging-drop preparations—one from a drop of dilute India-ink, a second from a culture of micrococci, and a third from a culture of the bacillus of typhoid fever. In what way do they differ?

STUDY OF SPORE-FORMATION.—The hanging-drop method just mentioned is not only employed for detecting the motility of an organism, but also for the study of its mode of spore-formation.

Since with aërobic organisms spore-formation occurs, as a rule, only in the presence of oxygen, and is induced more by limitation of the nutrition of the organisms than by any other factor, it is essential that these two points should be borne in mind in preparing the drop-cultures in which the process is to be studied. For this reason the drop of bouillon should be small and the air-chamber relatively large.

The cover-slip and hollow-ground slide should be carefully sterilized, and with a sterilized platinum loop a very small drop of bouillon is placed in the centre of the cover-slip. The slip is then inverted over the hollow depression in the sterilized object-glass and sealed with vaselin. The most convenient method of performing this last step in the process is to paint a ring of vaselin around the edges of the hollow in the slide, and then, without taking the cover-slip from the table upon which it rests, invert the hollow over the

drop and press it gently down upon the cover-slip. The vaselin causes the slip to adhere to the slide, so that it can be easily taken up. The drop now hangs in the centre of the small air-tight chamber which exists between the depression in the slide and the cover-slip. (See Fig. 38.)

A very thin drop of sterilized agar-agar may be substituted for the bouillon. It serves to retain the organisms in a fixed position, and the process may be more easily followed.

As soon as finished the preparation is to be examined microscopically and the condition of the organisms noted. It is then to be retained in a warm chamber especially devised for the purpose, and kept under continuous observation. The form of chamber best adapted to the purpose is one which envelops the whole microscope. It is provided with a window through which the light enters, and an arrangement by which the slide may be moved from the outside. The formation of spores requires a much longer time than the germination of spores into bacilli, but with patience both processes may be satisfactorily observed.

It will be noticed that the description of this process is very much like that which immediately precedes, but differs from it in one respect, viz., that in this manipulation we are not making a preparation which is simply to be examined and then thrown aside, but it is an actual pure culture, and must be kept as such, otherwise the observation will be worthless. For this reason the greatest care must be observed in the sterilization of all objects employed. Studies upon spore-formation by this method frequently continue over hours, and sometimes days, and contamination must, therefore, be care-

fully guarded against. The study should be begun with the vegetative form of the organisms; the hanging-drop preparation should, for this reason, always be made from a perfectly fresh culture of the organism under consideration before time has elapsed for spores to form.

The simple detection of the presence or absence of spore-formation can in many cases be made by other methods. For example, many species of bacteria which possess this property form spores most readily upon media from which it is somewhat difficult for them to obtain the necessary nourishment; potatoes and agar-agar that have become a little dry offer very favorable conditions, because of the limited area from which the growing bacteria can draw their nutritive supplies, and because of the free access which they have to oxygen, for, their growth being on the surface, they are surrounded by this gas unless means are taken to prevent it. By the hanging-drop method, however, more than this specific property may be determined. It is possible not only to detect the stages and steps in the formation of endogenous spores, but when the spores are completely formed their germination into mature rods may be seen by transferring them to a fresh bouillon-drop or drop of agar-agar preserved in the same way. The word rods is used because we have as yet no evidence that endogenous spore-formation occurs in any of the other morphological groups of bacteria.

Study of Gelatin Cultures.—As has been previously stated, the behavior of bacteria toward gelatin differs—some of them producing apparently no alteration in the medium, while the growth of others is accompanied by an enzymotic action that results in liquefaction of the gelatin at and around the place at

which the colonies are growing. In some instances this liquefaction spreads laterally and downward, causing a saucer-shaped excavation; while in others the colony sinks almost vertically into the gelatin and may be seen lying at the bottom of a funnel-shaped depression. These differences are constantly employed as one of the means of differentiating otherwise closely allied members of the same family of bacteria. (See Fig. 34.) Studies upon the spirillum of Asiatic cholera and a number of closely allied species, for example, reveal decided differences in the form of liquefaction produced by these various organisms. The minutest detail in this respect must be noted, and its frequency or constancy under varying conditions determined.

Cultures on Potato.—A very important feature in the study of an organism is its growth on sterilized potato. Many organisms present appearances under this method of cultivation which alone can almost be considered characteristic. In some cases coarsely lobulated, elevated, dry or moist patches of development occur after a few hours; again, the growth may be finely granular and but slightly elevated above the surface of the potato; at one time it will be dry and dull in appearance, again it may be moist and glistening. Sometimes bubbles, due to the fermentative action of the growing bacteria on the carbohydrates of the potato, are produced.

A most striking form of development on potato is that often exhibited by the bacillus of typhoid fever and the bacillus of diphtheria. After inoculation of a potato with either of these organisms there is usually no naked-eye evidence of growth, though microscopic examina-

tion of scrapings from the surface of the potato reveals an active multiplication of the organisms which had been planted there. The potato is one of the most important differential media that we possess for this work.

REACTIONS PRODUCED BY BACTERIA DURING THEIR GROWTH.—The reactions produced in media by different species of bacteria in the course of their growth are very valuable as means of differentiation. In some cases these changes are so marked that they are readily detected by the coarser reagents; again, they are so slight as to require the employment of the most delicate indicators. Bacteria sometimes produce at one period of their growth an alkaline, at another period an acid reaction. This is observed in cultures of the *bacillus diphtheriæ* of Löffler, when the media contain traces of fermentable carbohydrates.

These differences are best seen after the addition to the media in which the organisms are to grow of one of the chemical substances which do not interfere with the development of the organisms, but of which the color varies with the reaction, the change of reaction being indicated by the play of color. Such substances as litmus, in the form of the so-called "litmus tincture," and coralline (rosolic acid) in alcoholic solution, are commonly employed for this purpose. They may be added to the media in the proportions given in the chapter on media, and the changes in their colors studied with different bacteria. Milk and litmus tincture or peptone solution to which rosolic acid has been added are excellent media for this experiment.

In milk coagula now and then appear as a result of the influence of acids, produced from milk-sugar by

bacterial action, upon the casein of the milk, while at times acids may be produced and yet no coagulation take place.

ANILINE DYES FOR DIFFERENTIAL DIAGNOSIS.—The addition to solid media of some of the aniline dyes, fuchsin, methylene-blue, methylene-green, and several others, as well as combinations of these dyes, has been recommended as a means of differentiation of bacteria. The changes that are said to occur as a result of bacterial growth in media so treated consist of alterations in the color of the media due to the oxidizing or reducing properties of the growing bacteria. As yet, but little advantage has been derived from this method of work. It cannot at present be recommended as a reliable means of diagnosis.

BEHAVIOR TOWARD STAINING-REAGENTS.—The behavior of certain bacteria toward the different dyes and their reactions under special methods of after-treatment aid materially in their identification. With very few exceptions bacteria stain readily with the common aniline dyes; but they differ markedly in the tenacity with which they retain these colors under the subsequent treatment with decolorizing-agents.

The tubercle bacillus and the bacillus of leprosy, for example, are difficult to stain; but when once stained they retain their color under the action of such energetic decolorizing-agents as alcohol, nitric acid, oxalic acid, etc. Certain other organisms when stained with a solution of gentian-violet in aniline-water retain their color when treated with such decolorizing-bodies as iodine solution and alcohol (Gram's method), while again others are completely decolorized by this method. Many of them can only be washed in water, or but

for a few seconds in alcohol, without losing their color. It is essential that all these peculiarities should be carefully noted in studying an organism.

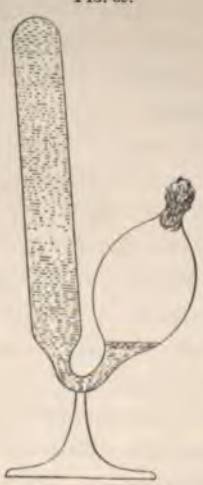
FERMENTATION.—The production of gas as an indication of fermentation is an accompaniment of the growth of certain bacteria. This is best studied in media to which 1 to 2 per cent. of grape-sugar (glucose) has been added. In this experiment the test-tube should be filled to about one-half with agar-agar. The medium is then liquefied, and when at the proper temperature a small quantity of a pure culture of the organism under consideration should be carefully distributed through it. The tube is then placed in ice-water and rapidly solidified in the vertical position. When solid it is placed in the incubator. After twenty-four to thirty-six hours, if the organism possesses the property of causing fermentation of glucose, the medium will be dotted everywhere with very small cavities containing the gas that has resulted.

This property of fermentation with evolution of gas is of such importance as a differential characteristic that latterly considerable attention has been given to it, and those who have been most intimately concerned in the development of our knowledge on the subject do not consider it sufficient to say that the growth of an organism "is accompanied by the production of gas-bubbles," but that under given conditions we should determine not only the amount of gas or gases produced by the organism under consideration, but also their nature. For this purpose Smith[1] recommends the

[1] An excellent and exhaustive contribution to this subject has been made by Theobald Smith in the Wilder Quarter-Century Book, Ithaca, N. Y., 1893.

employment of the fermentation-tube. This is a tube bent at an acute angle, closed at one end and enlarged with a bulb at the other. At the bend the tube is constricted. To it a glass foot is attached so that the tube may stand upright. (See Fig. 39.) To fill the tube the fluid (it is only used with fluid media)

FIG. 39.



Fermentation-tube.

is poured into the bulb until this is about half full. The tube is then tilted until the closed arm is nearly horizontal, so that the air may flow out into the bulb and the fluid flow into the closed arm to take its place. When this has been completely filled enough fluid should be added to cover the lowest expanding portion of the bulb, and the opening of the bulb plugged with cotton. The tubes thus filled are then to be sterilized. During sterilization they are to be maintained in the upright position. Under the influence of heat the

tension of the water-vapor in the closed arm forces most of the fluid into the bulb. As the tube cools the fluid returns to its place in the closed arm and fills it again, with the exception of a small space at the top, which is occupied by the air originally dissolved in the liquid and which has been driven out by the heat. The air-bubble should be tilted out after each sterilization; and finally, after the third exposure to steam, this arm of the tube will be free from air. The medium employed is bouillon containing some fermentable carbohydrate, as glucose, lactose, or saccharose. After inoculation the flasks are placed in the incubator, and the amount of gas that collects in the closed arm is noted from day to day. From studies that have been made this gas is found to consist usually of about one part by volume of carbonic acid and two parts by volume of an explosive gas consisting largely of hydrogen. For determining the nature and quantitative relations of these gases Smith[1] recommends the following procedure: "The bulb is completely filled with a 2 per cent. solution of sodium hydroxide (NaOH) and closed tightly with the thumb. The fluid is shaken thoroughly with the gas and allowed to flow back and forth from bulb to closed branch and the reverse several times, to insure intimate contact of the CO_2 with the alkali. Lastly, *before removing the thumb all the gas is allowed to collect in the closed branch*, so that none may escape when the thumb is removed. If CO_2 be present, a partial vacuum in the closed branch causes the fluid to rise suddenly when the thumb is removed. After allowing the layer of foam to subside somewhat the space occupied by gas is again measured, and the difference between this amount and that meas-

[1] Loc. cit., p. 196.

ured before shaking with the sodium hydroxide solution gives the proportion of CO_2 absorbed. The explosive character of the residue is determined as follows: the cotton plug is replaced and the gas from the closed branch is allowed to flow into the bulb and mix with the air there present. The plug is then removed and a lighted match inserted into the mouth of the bulb. The intensity of the explosion varies with the amount of air present in the bulb."

CULTIVATION WITHOUT OXYGEN.—As we have already learned, there is a group of bacteria to which the name "anaërobic organisms" has been given, which are characterized by inability to grow in the presence of free oxygen. For the cultivation of the members of this group a number of devices are employed for the exclusion of free oxygen from the cultures.

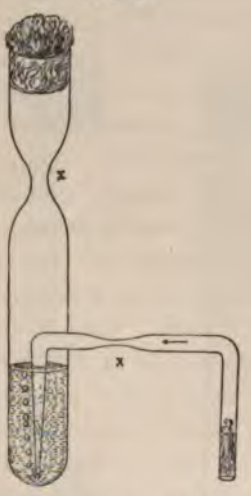
Koch's method. Koch covered the surface of a gelatin plate, which had been previously inoculated, with a thin sheet of sterilized isinglass. The organisms which grew beneath it were supposed to develop without oxygen.

Hesse's method. Hesse poured sterilized oil upon the surface of a culture made by stabbing a tube of gelatin. The growth that occurred along the track of the needle was supposed to be anaërobic in nature.

Methods of Liborius. Liborius has suggested two useful methods for this purpose. One is to nearly (about three-quarters) fill a test-tube with gelatin or agar-agar, which, after having been sterilized, is to be kept in a vessel of boiling water for ten minutes to expel all air from it. It is then rapidly cooled in ice-water, and when between 30° and 40° C., still fluid, is to be inoculated and very rapidly solidified. It is then sealed in

the flame. Anaërobic bacteria develop only in the lower layers of the medium. In his other method he employs a special tube, known as "the Liborius tube." Its construction is shown in Fig. 40.

FIG. 40.



Liborius tube for anaërobic cultures.

Through the side tube hydrogen is passed until it replaces all the air; the contracted parts, both of the neck of the tube and the side arm, are then sealed in the flame.[1] This tube can be used for either solid or liquid media, but, owing to its usual small capacity, gives better results with fluid media. (For precautions in

[1] As the tubes come from the maker the contracted parts marked x in the cut are usually so thick as to render the sealing in the flame during the passage of hydrogen somewhat troublesome; it is better to draw them out in the flame quite thin before passing the hydrogen to the tube. This makes the final sealing a matter of no difficulty.

using hydrogen, see note to Fränkel's method, page 210.)

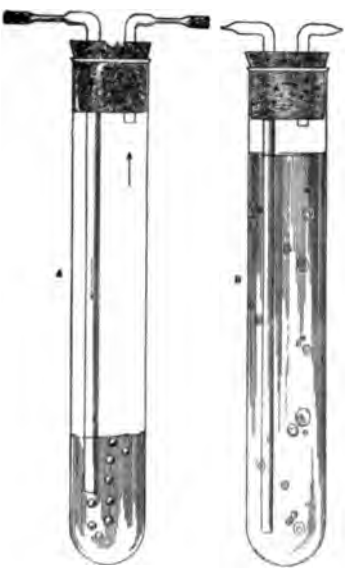
Method of Buchner. The plan suggested by Buchner, of allowing the cultures to develop in an atmosphere robbed of its oxygen by pyrogallic acid, gives very good results. In this method the culture, which is either a slant- or stab-culture in a test-tube, is placed—tube, cotton plug, and all—into a larger tube, in the bottom of which have been deposited 1 gramme of pyrogallic acid and 10 c.c. of $\frac{1}{10}$ normal [1] caustic-potash solution. The larger tube is then tightly plugged with a rubber stopper. The oxygen is quickly absorbed by the pyrogallic acid, and the organisms develop in the remaining constituents of the atmosphere, viz., nitrogen, a small amount of CO_2, and a trace of ammonia.

Method of C. Fränkel. Carl Fränkel suggests the following as a modification of or substitute for the tube of Liborius: the tube is first inoculated as if it were to be poured as a plate or rolled as an ordinary Esmarch tube. The cotton plug is then replaced by a rubber stopper, through which pass two glass tubes. These

[1] A normal solution is one that contains in a litre as many grammes of the dissolved substance as are indicated by its molecular equivalent. The equivalent is that amount of a chemical compound which possesses the same chemical value as does one atom of hydrogen. For example: one molecule of hydrochloric acid (HCl) has a molecular weight and also an equivalent weight of 36.5; a molecule of this acid has the same chemical value as one atom of hydrogen. Its normal solution is therefore 36.5 grammes to the litre. On the other hand, sulphuric acid (H_2SO_4) contains in each molecule two replaceable hydrogen atoms; its normal solution is not, therefore, 80 grammes (its molecular weight) to the litre, but that amount which would be equivalent chemically to one hydrogen atom, viz., 40 grammes (one-half its molecular weight) to the litre. A normal solution of caustic potash contains as many grammes to the litre as the number of its molecular weight—56.1 grammes to the litre of water.

must all have been sterilized in the steam sterilizer before using. On the outer side of the stopper these two tubes are bent at right angles to the long axis of the test-tube into which they are to be placed, and both are slightly drawn out in a gas-flame. Both of these

FIG. 41.



Fränkel's method for the cultivation of anaërobic bacteria.

tubes must be provided, before sterilization, with a plug of cotton; this is to prevent the access of foreign organisms to the medium during manipulations. At the inner side of the rubber stopper—that is, the end which is to be inserted into the test-tube—the glass tubes are of different lengths: one reaches to within 0.5 cm. of the bottom of the test-tube, the other is cut off flush with the under surface of the stopper. The

outer end of the longer glass tube is then connected with a hydrogen generator and hydrogen is allowed to bubble through the gelatin (Fig. 41, A) in the tube until all contained air has been expelled and its place taken by the hydrogen.[1] When the hydrogen has been bubbling through the gelatin for about five minutes (at least) one can be reasonably sure that all oxygen has been expelled. The drawn-out portions of the tubes can then be sealed in the gas-flame without fear of an explosion. The protruding end of the rubber stopper is then painted around with melted paraffin and the tube rolled in the way given for ordinary Esmarch tubes. A tube thus prepared and containing growing colonies is shown in Fig. 41, B.

The development that now occurs is in an atmosphere of hydrogen, all oxygen having been expelled. During the operation the tube containing the liquefied

[1] Before beginning the experiment it is always wise to test the hydrogen—*i. e.*, to see that it is free from oxygen and that there is no danger of an explosion, for unless this be done the entire apparatus may be blown to pieces and a serious accident occur. The agents used should be pure zinc and pure sulphuric acid of about 25 to 30 per cent. strength. With the primary evolution of the gas the outlet of the generator should be closed and kept closed until the gas reservoir is quite filled with hydrogen. The outlet should then be opened and the entire volume of gas allowed to escape, care being taken that no flame is in the neighborhood. This should be repeated, after which a sample of the hydrogen generated should be collected in an inverted test-tube in the ordinary way for collecting gases over water, viz., by filling a test-tube with water, closing its mouth with the thumb, inverting it, and placing its mouth under water, when, after removing the thumb, the water will be kept in it by atmospheric pressure. The hydrogen which is flowing from the open generator may be conducted to the test-tube by rubber tubing. When the water has been replaced test the gas by holding a flame near the open mouth of the test-tube. If no explosion occurs, the hydrogen is safe to use. Should there be an explosion, the generation of hydrogen must be continued in the apparatus until it burns with a colorless flame when tested in a test-tube.

gelatin should be kept in a water-bath at a temperature sufficiently high to prevent its solidifying, and at the same time not high enough to kill the organisms with which it has been inoculated.

One of the obstacles to the successful performance of this method is the bubbling of the gelatin, the foam from which will often fill the exit-tube and sometimes be forced from it. This may be obviated by reversing the order of proceeding, viz.: roll the Esmarch tube in the ordinary way with the organisms to be studied, using a relatively small amount of gelatin, so as to have as thin a layer as possible when it is rolled. Then replace the cotton plug with the sterilized rubber stopper carrying the glass tubes through which the hydrogen is to be passed, and allow the hydrogen to flow through as in the method first given. The gas now passes *over* the gelatin instead of *through* it, and consequently no bubbling results. In all other respects the procedure is the same as that given by Fränkel.

Method of Kitasato and Weil. For favoring anaërobic conditions Kitasato and Weil have suggested the addition to the culture-media of some strong reducing-agent. They recommend formic acid or sodium formate in 0.3 to 0.5 per cent.; glucose in 1.5 to 2 per cent.; or blue litmus tincture in 5 per cent. by volume. This is, of course, in addition to an atmosphere from which all oxygen has been expelled. As a reducing-agent for this purpose Theobald Smith regards a weaker solution of glucose, 0.3 to 0.5 per cent., as more advantageous.

Esmarch's method. Esmarch's plan is to prepare in the usual way a roll-tube of the organisms; subject it

to a low temperature, and while quite cold fill it with liquefied gelatin, which is caused to solidify rapidly. In this method the colonies develop along the sides of the tubes, and can be more easily studied than when they are scattered through the gelatin, as in the method of Liborius.

By some workers the oxygen is removed from the culture-medium by the use of the air-pump.

Many other methods are employed for this special purpose, but for the beginner those given will suffice.

From what has been said it may be inferred that the cultivation of anaërobic bacteria is a simple matter and attended with but little difficulty. Such an inference will, however, be quickly abandoned when the beginner attempts this part of his work for the first time, and particularly when his efforts are directed toward the separation of these forms from other organisms with which they are associated. The presence of spore-forming, *facultative* anaërobes in mixed cultures is always to be suspected, and it is this group that renders the task so difficult. At best the work requires undivided attention and no small degree of skill in bacteriological technique.

Indol Production.—The generation of products other than those that give rise to alterations in the reaction of the media, and whose presence may be detected by chemical reactions, is now a recognized step in the identification of different species of bacteria. Among these products is one that is produced by a number of organisms, and whose presence may easily be detected by its characteristic behavior when treated with certain substances. I refer to *nitroso-indol*, the reactions of which were described by Beyer in 1869, and

the presence of which as a product of the growth of certain bacteria has since furnished a topic for considerable discussion.

Indol, the name by which this body is now generally known, when acted upon by reducing-agents becomes of a more or less decided rose color. This body was recognized as one of the products of growth of the spirillum of Asiatic cholera first by Poel, and a short time subsequently by Bujwid and by Dunham, and for a time was believed to be peculiarly characteristic of the growth of this organism. It has since been found that there are many other bacteria which also possess the property of producing indol in the course of their development. It is constantly present in putrefying matters, and is one of the aromatic bodies that give to fæces their characteristic odor.

The methods employed for its detection are as follows: cultivate the organism for twenty-four to forty-eight hours at a temperature of 37° C., in the simple peptone solution known as "Dunham's solution" (see formula for this medium). This solution is preferred because its pale color does not mask the rose color of the reaction when the amount of indol present is very small.

Four tubes should always be inoculated and kept under exactly the same conditions for the same length of time.

At the end of twenty-four or forty-eight hours the test may be made. Proceed as follows: to a tube containing 7 c.c. of the peptone solution, but which has *not* been inoculated, add 10 drops of concentrated sulphuric acid. To another similar tube add 1 c.c. of a 0.01 per cent. solution of sodium nitrite, and afterward 10 drops

of concentrated sulphuric acid. Observe the tubes for five to ten minutes. No alteration in their color appears, or at least there is no production of a rose color. They contain no indol.

Treat in the same way, with the acid alone, two of the tubes which *have been inoculated*. If no rose color appears after five or ten minutes, add 1 c.c. of the sodium nitrite solution. If now no rose color is produced, the indol reaction may be considered as negative —*i. e.*, no indol has been formed as a product of the growth of the bacteria.

If indol is present, and the rose color appears after the addition of the acid alone, it is plain that not only indol has been formed, but coincidently a reducing-body. This is found, by proper means, to be nitrous acid. The sulphuric acid liberates this acid from its salts and permits of its reducing action being brought into play.

If the rose color appears only after the addition of both the acid and the nitrite solution, then indol has been formed during the growth of the organisms, but no nitrites.

Control the results obtained by treating the two remaining cultures in the same way.

The test is sometimes made by allowing concentrated sulphuric acid to flow down the sides and collect at the bottom of the tube; the reaction is then seen as a rose-colored zone overlying the line of contact of the acid and culture-medium. This method is open to the objection that, if indol is present in only a very small amount, the faint rose tint produced by it is apt to be masked by a brown color that results from the charring action of the concentrated acid on the other organic matters

in the culture-medium, so that its presence may in this way escape detection. In view of this, Petri recommends the use of *dilute* sulphuric acid. He states that when indol is present the characteristic rose color appears a little more slowly with the dilute acid, but it is more permanent, and there is never any likelihood of its presence being masked by other color-reactions.

Muir and Ritchie recommend the use of ordinary fuming or yellow nitric acid for this test. In this method two or three drops of the acid are added to the culture under consideration. If indol be present, the red color appears as a result of the reducing action of the nitrous acid upon it. The defect in this method is that it reveals the presence of only indol, and does not indicate whether or not reducing-bodies were coincidently formed with the indol. As a test for indol alone it is convenient and entirely trustworthy.

Test for nitrites. For this purpose Lunkewicz has recently recommended the employment of Ilosvay's modification of the method of Griess. As reagents the following solutions are employed:

a. Naphthylamine	0.1 gramme.
Distilled water	20.0 c.c.
Acetic acid (25 per cent. sol.)	150.0 c.c.
b. Sulfanilic acid	0.5 gramme.
Acetic acid (25 per cent. sol.)	150.0 c.c.

In preparing solution *a* the naphthylamine is dissolved in 20 c.c. of boiling water, filtered, allowed to cool, and mixed with the dilute acetic acid. Solutions *a* and *b* are then mixed. The resulting mixture should be colorless. It is best to prepare it as needed, though if kept in a closely stoppered flask it retains its virtues for some time.

When added to cultures containing nitrites, in the proportion of one volume of reagent to five volumes of the culture, a deep-red color appears in a few seconds. If no nitrites are present, no color-reaction occurs. In testing cultures always control the results by tests on a portion of the same medium *not* inoculated, as some of the ingredients of which the medium is composed may contain nitrites. Lunkewicz recommends the use of Merck's peptone for this test, claiming that nitrites are always to be found in Witte's peptones.

POINTS TO BE OBSERVED IN DESCRIBING AN ORGANISM.

The following is an outline of points to be considered in describing a new organism or in identifying an organism with one already described:

1. Its source—as air, water, or soil. If found in the animal body, is it normally present or only in pathological conditions?

2. Its form, size, mode of development, occurrence of involution-forms or other variations in morphology. Grouping, as in pairs, chains, clumps, zoöglœa; presence of capsule; development and germination of spores; arrangement of flagella.

3. Staining-peculiarities—especially its reactions with Gram's (or Weigert's fibrin) stain, and peculiar or irregular modes of staining.

4. Motility—to be determined on very fresh cultures and on cultures in different media.

5. Its relation to oxygen—Is it aërobic, anaërobic, or facultative? Does it develop in other gases, as carbonic acid, hydrogen, etc.?

6. Both the macroscopic and microscopic appearance of its colonies on nutrient gelatin and on nutrient agar-agar.

7. The appearance of its growth in stab- and slant-cultures on gelatin, agar-agar, blood-serum, and potato.

8. The character of its growth in fluid media, as in bouillon, milk, litmus-milk, rosolic-acid-peptone solution, and in bouillon containing glucose.

9. Does it grow best in acid, alkaline, or neutral media?

10. Is the normal reaction of the medium altered by its growth? Is its growth accompanied by the production of indol? Is the indol associated with the coincident production of nitrites?

11. Is its growth accompanied by the production of gas, as evidenced by the appearance of bubbles in the media—both in media containing fermentable sugars and those from which these bodies are absent? When cultivated in sugar-bouillon in the fermentation-tube, what proportion of gas is evolved under known conditions? How much of this gas is carbonic acid, and how much is explosive?

12. At what temperature does it thrive best, and what are the lowest and highest temperatures at which it will develop? What is its thermal death-point, both by steam and dry-air methods of determining this point?

13. What is its behavior when exposed to chemical disinfectants and antiseptics? Does it withstand drying and other injurious influences, both in the vegetative and spore stages? The germicidal activity of the blood-serum of different animals may also be tried upon it.

14. Its pathogenic powers—modes of inoculation by

which these are demonstrated; quantity of material used in inoculation; duration of the disease and its symptoms; lesions produced, and distribution of the bacteria in the inoculated animals; which animals are susceptible and which immune, and the character of its pathogenic activities. Variations in virulence, and the probable causes to which they are due. Can they be produced artificially and at will?

15. The detection of specific, toxic, and immunizing products of growth.

16. Its behavior when exposed to the influence of blood-serum of animals immunized from it; also its behavior when mixed with serum from an animal in the acute stage of infection resulting from it. Are the relations between the organism and the serum constant and specific?

CHAPTER XII.

Inoculation of animals—Subcutaneous inoculation—Intravenous injection—Inoculation into the great serous cavities, and into the anterior chamber of the eye—Observation of animals after inoculation.

After subjecting an organism to the methods of study that we have thus far reviewed there remains to be tested its action upon animals—*i. e.*, to determine if it possesses the property of producing disease or not; and, if so, what are the pathological results of its growth in the tissues of these animals, and in what way must it gain entrance to the tissues in order to produce these results? The mode of deciding these points is by inoculation, which is practised in different ways according to circumstances. Most commonly a bit of the culture to be tested is simply introduced beneath the skin of the animal; but in other cases it may be necessary to introduce it directly into the vascular or lymphatic circulation, or into one or the other of the great serous cavities; or, for still other purposes of observation, into the anterior chamber of the eye, upon the iris.

Subcutaneous Inoculation of Animals.—The animals usually employed in the laboratory for purposes of inoculation are white mice, gray house-mice, guinea-pigs, rabbits, and pigeons.

For simple subcutaneous inoculation the steps in the process are practically the same in all cases. The hair or feathers are to be carefully removed. If the skin is very

dirty, it may be scrubbed with soap and water. Sterilization of the skin is practically impossible, so it need not be attempted. If the inoculation is to be made by means of a hypodermic syringe, then a fold of the skin may be lifted up and the needle inserted in the usual way. If a solid culture is to be inoculated, a fold of skin may be taken up with forceps and a pocket cut into it with scissors which have previously been sterilized. This pocket must be cut large enough to admit the end of the needle without its touching the sides of the opening as it is inserted. Beneath the skin will be found the superficial and deep connective-tissue fasciæ. These must be taken up with sterilized forceps, and with sterilized scissors incised in a way corresponding to the opening in the skin. The pocket is then to be held open with the forceps and the substance to be inserted is introduced as far under the skin and fasciæ as possible, care being taken not to touch the edges of the wound if it can be avoided. The edges of the wound may then be simply pulled together and allowed to remain. No stitching or efforts at closing it are necessary, though a drop of collodion over the point of operation may serve to lessen contamination.

During manipulation the animal must be held still. For this purpose special forms of holders have been devised; but if an assistant is at hand, the simple subcutaneous inoculation may be made without the aid of a mechanical holder.

It is at times, however, more convenient to dispense with an assistant; one of several forms of apparatus that have been devised for holding mice, guinea-pigs, rats, rabbits, etc., may then be used. For small animals, such as mice and rats, the holder suggested by Kitasato is very

useful. It is simply a metal plate attached to a stand by a clamped ball-and-socket joint, so that it can be fixed in

FIG. 42.

Kitasato's mouse-holder.

any position. It is provided with a spring-clip at one end that holds the animal by the skin of the neck, and

FIG. 43.

Holder for larger animals.

at the other end with another clamp that holds the tail of the animal. This holder is shown in Fig. 42. For

larger animals the form of holder shown in Fig. 43 is commonly used.

A very simple and useful holder for guinea-pigs consists of a metal cylinder of about 5 cm. diameter and

FIG. 44.

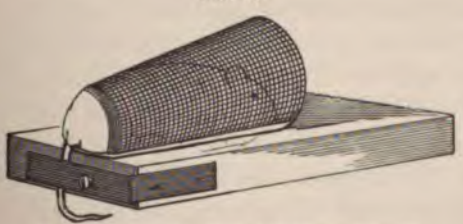
The Voges holder for guinea-pigs.

about 13 cm. long, closed at one end by a perforated cap of either tin or wire netting. Along the side of this box is a longitudinal slit 12 mm. wide that runs for 9.5 cm. from within 0.5 mm. of the open extremity of the cylinder. The animal is placed in such a cylinder

with its head toward the perforated bottom. It is then easily possible to make subcutaneous inoculation by taking up a bit of skin through the slit in the side of the box, or to make intraperitoneal injection by drawing the posterior extremities slightly from the box and holding them steady between the index and second finger, as seen in Fig. 44. It is also very convenient for use when the rectal temperature of these small animals is to be taken. The manipulations can easily be made without the aid of an assistant. Its construction is seen in Fig. 44.[1]

For ordinary subcutaneous inoculations at the root of the tail in mice a simple apparatus consists of a piece of board about 7 x 10 cm. and 2 cm. thick, upon which is tacked a hollow truncated cone of wire gauze, about 6 cm. long and about 1.5 cm. in diameter at one end and 2 cm. at its other end. This is tacked

FIG. 45.



Mouse-holder, with mouse in proper position.

upon the board in such a position that its long axis is in the long axis of the board, being equidistant from its sides. Its small end is placed at the edge of the board. The mouse is taken up by the tail by means of a pair

[1] Centralblatt für Bakteriologie und Parasitenkunde, 1895, Bd. xviii. p. 530.

of tongs and allowed to crawl into the smaller end of the wire cone. When so far in that only the root of the tail projects the animal is fixed in this position by a clamp and thumb-screw, with which the apparatus (Fig. 45) is provided. The animal usually remains perfectly quiet and may be handled without difficulty.

The hair over the root of the tail is to be carefully cut away with scissors and a pocket cut through the skin at this point. The inoculation is then made into the loose tissue under the skin over this part of the back in the way that has just been described. It is always best to insert the needle some distance along the spinal column, and thus deposit the material as far from the surface-wound as possible.

As the subcutaneous inoculation is very simple and takes only a few moments, guinea-pigs, rabbits, and pigeons may be held by an assistant. The front legs in the one hand and the hind legs in the other, with the animal stretched upon its back on a table, is the usual position for the operation when practised upon guinea-pigs and rabbits. The point at which the inoculations are commonly made is in the abdominal wall, either to the right or left of the median line and about 3 cm. distant. When pigeons are used they are held with the legs, tail, and ends of the wings in the one hand, and the head and anterior portion of the body in the other, leaving the area occupied by the pectoral muscles, over which the inoculation is to be made, free for manipulation. The hair over the point selected for the inoculation should be closely cut with scissors in the case of guinea-pigs and rabbits, and from a small area the feathers should be plucked in the case of the pigeon.

Injection into the Circulation.—It is not in-

frequently desirable to inject the material under consideration directly into the circulation of an animal. If a rabbit is employed for the purpose, the operation is usually done upon one of the veins in the ear. To those who have had no practice with this procedure it offers a great many difficulties; but if the directions which will be given are strictly observed, the greatest of these obstacles to the successful performance of the operation may be overcome.

When viewing the circulation in the ear of the rabbit by transmitted light three conspicuous branches of the main vessel (*vena auricularis posterior*) will be seen. One runs about centrally in the long axis of the ear, one runs along its anterior margin, and one along its posterior margin. The central branch (*ramus anterior* of the *vena auricularis posterior*) is the largest and most conspicuous vessel of the ear, and is, therefore, believed by the inexperienced to be the branch into which it would appear easiest to insert a hypodermic needle. This, however, is fallacious. This vessel lies very loosely imbedded in connective tissue, and, in efforts to introduce a needle into it, rolls about to such an extent that only after a great deal of difficulty does the experiment succeed. On the other hand, the posterior branch (*ramus lateralis posterior* of the *vena auricularis posterior*) is a very fine, delicate vessel which runs along the posterior margin of the ear, and is so firmly fixed in the dense tissues which surround it that it is prevented from rolling about under the point of the needle. The further away from the mouth of the vessel—that is, the nearer we approach its capillary extremity—the more favorable become the conditions for the success of the operation.

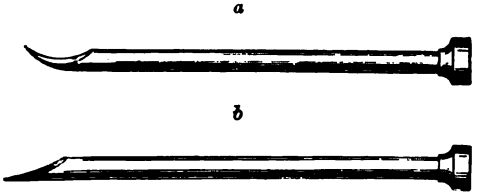
Select, then, the very delicate vessel lying quite close to the posterior margin of the ear, and make the injection as near to the apex of the ear as possible. At times the vessels of the ear will be found to contain so little blood that they are hardly distinguishable, making it very difficult to introduce the needle into them. This is sometimes overcome by pressure at the root of the ear, causing stasis of the blood and distention of the vessels. A very satisfactory method of causing the veins to become prominent is to press lightly or prick gently with the point of a needle the skin over the vessel to be used. In a few seconds, as a result of this irritation, the vessel will have become distended with blood, and readily distinguishable from the surrounding tissue; it may then be easily punctured by the needle of the syringe. A sharp flick with the finger will often produce the same result. The injection is always to be made from the dorsal surface of the ear.

Of no less importance than the selection of the proper vessel is the shape of the point of the needle employed. The hypodermic needles as they come from the makers are not suited at all for this operation, because of the manner in which their points are ground. If one examine carefully the point of a new hypodermic needle, it will be seen that the long point, instead of presenting a *flat*, slanting surface when viewed from the side, has a more or less *curved* surface. Now, in efforts to introduce such a needle into a vessel of very small calibre it is usually seen that the point of the needle, instead of remaining in the vessel, as it would do were it straight, very commonly projects into the opposite wall; and as the needle is inserted further and further it is usually pushed through the vessel-walls into the

loose tissues beyond, and the material to be injected is deposited in these tissues, instead of into the circulation. If, on the contrary, the slanting point of the needle be ground until its surface is perfectly flat when viewed from the side, and no curvature exists, then when once inserted into a vessel it usually remains there, and there is no tendency to penetrate the opposite wall. We never use a new hypodermic needle until its point is carefully ground to a perfectly flat, slanting surface with no curvature whatever.

These differences may perhaps be more easily understood if represented diagrammatically. In Fig. 46, *a*,

FIG. 46.



Hypodermic needles, magnified. *a*. Improper point. *b*. Proper shape of point.

the needle has the point usually seen when new. In Fig. 46, *b*, the point has been ground to the shape best suited for this operation. The needles need not be returned to the maker. One can grind them to the shape desired in a few minutes upon an oilstone. The size of the needle is that commonly employed by physicians for subcutaneous injections in human beings.

When the operation is to be performed an assistant holds the animal gently but firmly in the crouching position upon a table. If the animal does not remain quiet, it is best to wrap it in a towel, so that only its head protrudes; though in most cases we have not

found this necessary, particularly if the animal has not been excited prior to beginning the operation.

The ear in which the injection is to be made is to be shaved clean of hair by means of a razor and soap.

The animal should be placed so that the prepared ear comes between the operator and the source of light. This renders visible by transmitted light not only the coarser vessels of the ear, but also their finer branches.

The filled hypodermic syringe is taken in one hand and with the other hand the ear is held firmly. The point of the needle is then inserted through the skin and into the finest part of the *ramus posterior*, the part nearest the apex of the ear, where the course of the vessel is nearly straight. When the point of the needle is in this vessel it gives to the hand a sensation quite different from that felt when it is in the midst of connective tissue. As soon as one supposes the point of the needle is in the vessel a drop or two of the fluid may be injected from the syringe, and, if his suspicions are correct, the circulation in the small ramifications and their anastomoses will rapidly alter in appearance—*i. e.*, the circulating blood will be very quickly replaced by the clear transparent fluid that is being injected. At this stage one must proceed very carefully, for sometimes when the needle-point is not actually in the vessel, but is in the lymph-spaces surrounding it, an appearance somewhat similar is seen. This may always be differentiated, however, by continuing the injection, when the flow of clear fluid through the vessels will not only fail to take the place of the circulating blood, but at the same time a localized swelling, due to an accumulation of the fluid injected, will appear under the skin about the point of the needle. The needle must

then be withdrawn and inserted into the vessel at a point a little nearer its proximal end.

Care must be taken that no air is injected.

The hypodermic syringe and needle must, previous to operation, have been carefully sterilized in the steam sterilizer or in boiling water. The animal must be kept under close observation for about an hour after injection.

The operation is one that cannot be learned from verbal description. It can only be successfully performed after actual practice. If the precautions which have been mentioned are observed, but little difficulty in performing the operation will be experienced.

Its greater convenience and simplicity, as compared with other methods for the introduction of substances into the circulation, commend it as a technical procedure with which to make one's self familiar. The animals sustain practically no wound, they experience no suffering—at least they give no evidence of pain—and no anæsthetic is required.

The form of syringe best suited for this operation is of the ordinary design, but one that permits of thorough sterilization by steam. It should be made of glass and metal, with packings that may be sterilized by steam without injury. The syringes commonly employed are those shown in Fig. 47.

For operations requiring exact dosage experience has led me to prefer a syringe after the pattern of *C*, in Fig. 47—*i. e.*, the form commonly used by physicians. The reason for this is as follows: in making injections, either into the circulation or under the skin, there is a certain amount of resistance to the passage of fluid from the needle. If one overcomes this resistance

by means of a cushion of compressed air, as is the case in syringes *A* and *B*, Fig. 47, the sudden expansion of the air in the body of the syringe when resistance is overcome frequently causes a larger amount of fluid to be ejected than is desired. No such accident is likely

FIG. 47.

Forms of hypodermic syringe.
A. Koch's syringe. *B*. Syringe of Strohschein. *C*. Overlack's form.

to occur when the fluid is forced from the barrel of the syringe by the head of a close-fitting piston, with no air intervening between the fluid and the head of the piston. With such an instrument, properly manipulated, the dose can always be controlled with accuracy.

INOCULATION INTO THE LYMPHATIC CIRCULATION. —Fluid cultures or suspensions of bacteria may be injected into the lymphatics by way of the testicles. The operation is in no wise complicated. One simply plunges the point of the hypodermic needle directly into the substance of the testicle and then injects the amount desired.

ons made in this manner are sometimes followed
ng pathological lesions of the lymphatic
he abdomen.

Inoculation into the Great Serous Cavities. —Inoculation into the *peritoneum* presents no difficulties if fluids are to be introduced. In this case one makes, with a pair of sterilized scissors, a small nick through the skin down to the underlying fasciæ, and, taking a fold of the abdominal wall between the fingers, plunges the hypodermic needle through the opening just made directly into the peritoneal cavity. There is little or no danger of penetrating the intestines or other internal viscera if the puncture be made along the median line at about midway between the end of the sternum and the symphysis pubis. Though this may seem a rude method, it is rarely that the intestines are penetrated. The object of the primary incision is to lessen the chances of contamination by bacteria located in the skin, some of which would adhere to the needle if it were plunged directly through the skin, and might complicate the results.

If solid substances, bits of tissue, etc., are to be introduced into the peritoneum, it becomes necessary to conduct the operation upon the lines of a laparotomy. The hair should be shaved from a small area over the median line, after which the skin is to be thoroughly washed. A short longitudinal incision (about 2 cm. long) is then to be made in the median line through the skin and down to the fasciæ. Two subcutaneous sutures, as employed by Halsted, are then to be introduced transversely to the line of incision about 1 cm. apart, and their ends left loose. This particular sort of suture does not pass through the skin, but, instead, the needle is introduced into the subcutaneous tissues along the edge of the incision. In this case they are to pass into the abdominal cavity and out again, entering at one

side of the line of incision and leaving at the other, as indicated by the solid and dotted lines in Fig. 48. (The figure indicates the primary opening through the skin. The longitudinal dotted line shows the opening to

FIG. 48.

Diagram of skin incision and sutures in laparotomy on animals.

be made into the abdomen; the transverse dotted lines, with their loose ends, represent the sutures as placed in position *before the abdomen is opened;* it will be seen that these sutures in all cases pass through the subcutaneous tissues *only* and do not penetrate the skin proper.)

The opening through the remaining layers may now be completed; the bit of tissue is deposited in the peritoneal cavity, under precautions that will exclude all else, the edges of the wound drawn evenly and gently together by tying the sutures, and the lines of incision dressed with collodion. It should be needless to say that this operation must be conducted under the strictest precautions, to avoid complications. All instruments, sutures, ligatures, etc., must be carefully sterilized either

in the steam sterilizer for twenty minutes, or by boiling in 2 per cent. sodium carbonate solution for ten minutes; the hands of the operator, though they should not touch the wound, must be carefully cleansed, and the material to be introduced into the abdomen should be handled with only sterilized instruments.

Inoculation into the *pleural* cavity is much less frequently required—in fact, it is not a routine method. It is not easy to enter the pleural cavity with a hypodermic needle without injuring the lung, and it is rare that conditions call for the introduction of solid particles into this locality.

Inoculation into the anterior chamber of the eye is performed by making a puncture through the cornea just in front of its junction with the sclerotic, the knife being passed into the anterior chamber in a plane parallel to the plane of the iris. By the aid of a fine pair of forceps the bit of tissue is passed through the opening thus made and is deposited upon the iris, where it is allowed to remain, and where its pathogenic activities upon the iris can be conveniently studied. It is a mode of inoculation of very limited application, and is therefore but rarely practised. It was employed in the classical experiments of Cohnheim in demonstrating the infectious nature of tuberculous tissues, tuberculosis of the iris being the constant result of the introduction of tuberculous tissue into the anterior chamber of the eye of rabbits.

Observation of Animals after Inoculation. —After either of these methods of inoculation, particularly when unknown species of bacteria are being tested, the animal is to be kept under constant observation and all deviations from the normal are to be carefully noted

—as, for instance, elevation of temperature; loss of weight; peculiar position in the cage; loss of appetite;

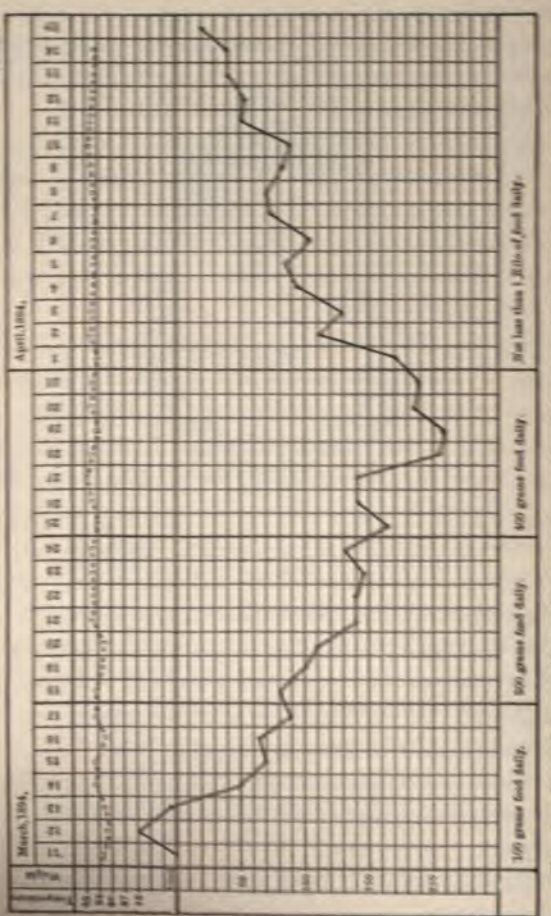


FIG. 40.

Showing diurnal fluctuations of temperature and weight of a rabbit receiving varying amounts of food. Animal otherwise under normal conditions.

roughening of the hair; excessive secretions, from either r-passages, conjunctiva, or kidneys; looseness of or hage from the bowels; tumefaction or reaction at

site of inoculation, etc. If death ensue in from two to four days, it may reasonably be expected that at autopsy

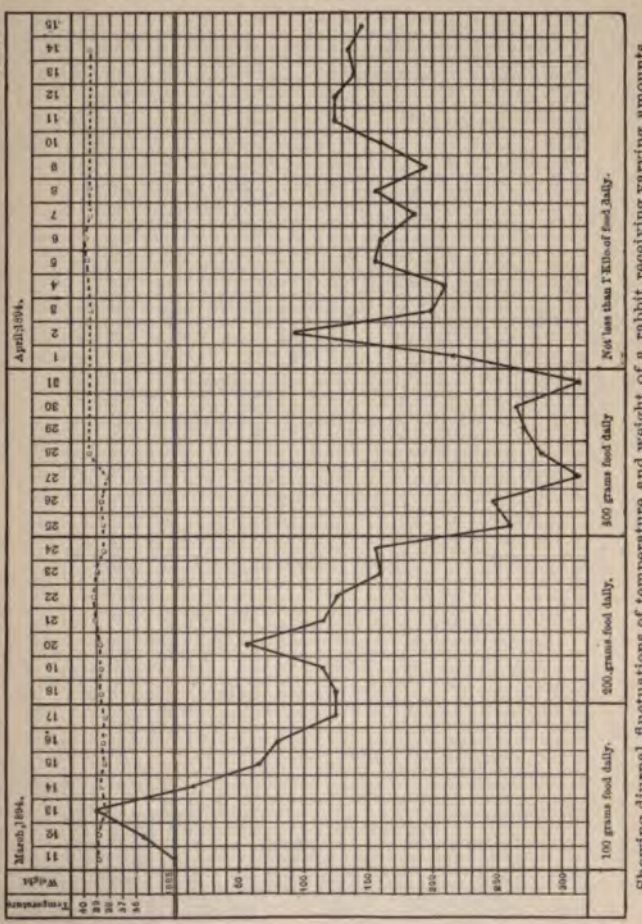


FIG. 50.

Showing diurnal fluctuations of temperature and weight of a rabbit receiving varying amounts of food. Animal otherwise under normal conditions.

evidence of either acute septic or toxic processes will be found. It sometimes occurs, however, that inoculation results in the production of chronic conditions, and the

animal must be kept under observation often for weeks. In these cases it is important to note the progress of

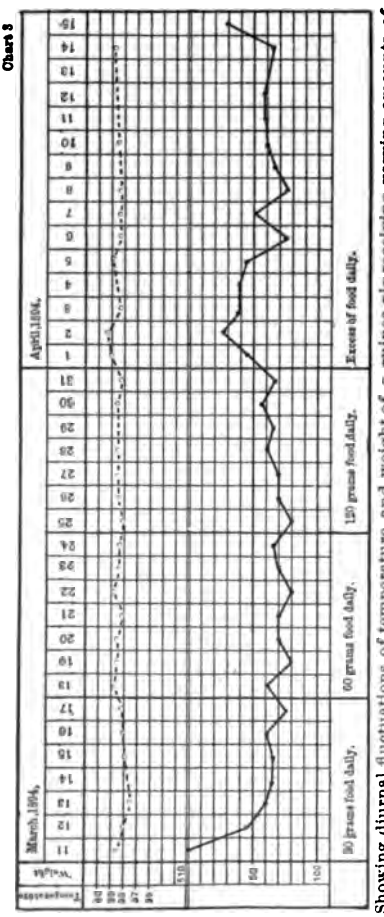


FIG. 51.

Showing diurnal fluctuations of temperature and weight of a guinea-pig receiving varying amounts of food. Animal otherwise under normal conditions.

the disease by its effect upon the physical condition of the animal, viz., upon the nutritive processes, as evi-

denced by fluctuation in weight, and upon the body-temperature. For this purpose the animal is to be

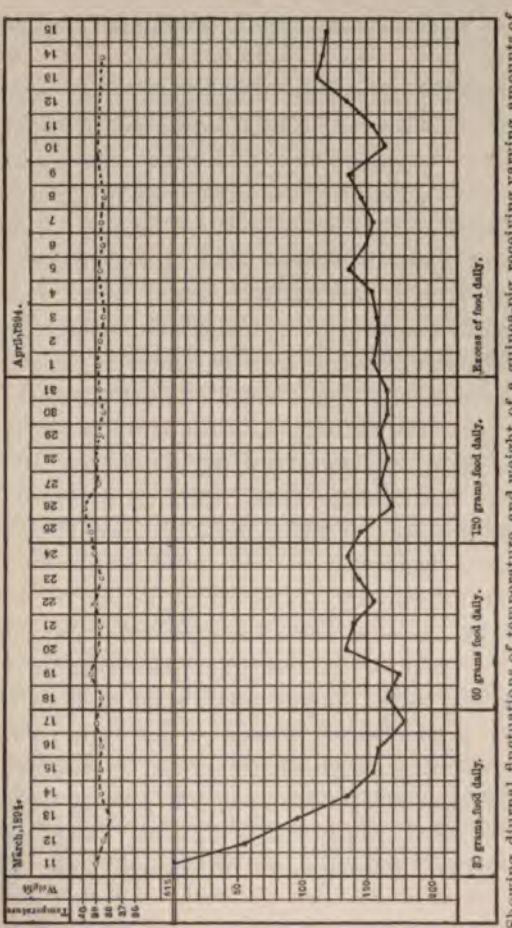


FIG. 52.

Showing diurnal fluctuations of temperature and weight of a guinea-pig receiving varying amounts of food. Animal otherwise under normal conditions.

weighed daily, always at about the same hour and always about midway between the hours of feeding;

at the same time its temperature, as indicated by a thermometer placed in the rectum, is to be recorded.[1] By comparison of these daily observations the observer is aided in determining the course the infection is taking.

Too much stress must not, however, be laid upon moderate and sudden daily fluctuations in either temperature or weight, as it is a common observation that presumably normal animals when confined in cages and fed regularly often present very striking temporary gains and losses in weight, often amounting to 50 or 100 grammes in twenty-four hours, even in animals whose total weight may not exceed 500 or 600 gammes; similarly unexplainable rises and falls of temperature, often as much as a degree from one day to another, are seen. Such fluctuations have apparently no bearing upon the general condition of the animal, but are probably due to transient causes, such as overfeeding or scarcity of food, improper feeding, lack of exercise, excitement, fright, etc.

The accompanying charts (Figs. 49, 50, 51, 52) will serve to illustrate some of these points. The animals, two rabbits and two guinea-pigs, were taken at random from among stock animals and placed each in a clean cage, the kind used for animals under experiment, and kept under as good general conditions as possible. For the first week the rabbits received each 100 grammes of green food (cabbage and turnips) daily, and the guinea-pigs 30 grammes each of the same food. During

[1] The thermometer must be inserted into the rectum beyond the grasp of the sphincter, otherwise pressure upon its bulb by contraction of this muscle may force up the mercurial column to a point higher than that resulting from the actual body-temperature.

the second week this daily amount of food was doubled; during the third week it was quadrupled; and for the fourth and fifth weeks they each received an excess of food daily, consisting of green vegetables and grains (oats and corn). By reference to the charts sudden diurnal fluctuations in weight will be observed that do not correspond in all instances with scarcity or sufficiency of food. With the rabbits there is a gradual loss of weight with the smaller amounts of food, which losses are not totally recovered as the food is increased. With the guinea-pigs there is likewise at first a loss; but after a short time the weight remains tolerably constant, and is not so conspicuously affected by the increase in food as one might expect. From the recorded temperatures one sees the peculiar fluctuations mentioned. To just what they are due it is impossible to say. It is manifest that the normal temperature of these animals, if we can speak of a normal temperature for animals presenting such fluctuations, is about a degree or more, Centigrade, higher than that of human beings. The animals from which these charts were made were not inoculated, nor were they subjected to any operative procedures whatever, the only deviations from normal conditions being the variations in the daily amount of food given.

In certain instances, however, there will be noticed a constant tendency to diminution in weight, notwithstanding the daily fluctuations, and after a time a condition of extreme emaciation may be reached, the animal often being reduced to from 50 to 60 per cent. of its original weight. In other cases, after inoculations to which the animal is not susceptible, rabbits in particular, if properly fed, will frequently gain steadily in

weight. The condition of progressive emaciation just mentioned is conspicuously seen after intravenous inoculation of rabbits with cultures of *bacillus typhi abdominalis* and of *bacterium coli commune*, referred to in the chapter on the latter organism, and if looked for will doubtless be seen to follow inoculation with other organisms capable of producing chronic forms of infection, but which are frequently considered non-pathogenic because of their inability to induce acute conditions. Not infrequently in chronic infections there may be hardly any marked and constant temperature-variations until just before death, when sometimes there will be a rise and at other times a fall of temperature. In the majority of cases, however, one must be very cautious as to the amount of stress laid upon changes in weight and temperature, for unless they are progressive or continuous in one or another direction they may have little or no significance as indicating the existence or absence of disease.

CHAPTER XIII.

Post-mortem examination of animals—Bacteriological examination of the tissues—Disposal of tissues and disinfection of instruments after the examination—Study of tissues and exudates during life.

During bacteriological examination of the tissues of dead animals certain precautions must be rigidly observed in order to arrive at correct conclusions.

The autopsy should be made as soon as possible after death. If delay cannot be avoided, the animal should be kept on ice until the examination can be made, otherwise decomposition sets in, and the saprophytic bacteria now present may interfere with the accuracy of results. When the autopsy is to be made the animal is first inspected externally, and all visible lesions noted. It is then to be fixed upon its back upon a board with nails or tacks. The four legs and the end of the nose, through which the tacks are driven, are to be moderately extended. Plates are now to be made from the site of inoculation, if this is subcutaneous. The surfaces of the thorax and abdomen are then to be moistened to prevent the fine hairs, dust, etc., from floating about in the air and interfering with the work. An incision is then made through the skin from the chin to the symphysis pubis. This is only a skin incision, and does not reach deeper than the muscles. It is best done by first making with a scalpel an incision just large enough to permit of the introduction of one blade of a blunt-pointed scissors. It is

then completed with the scissors. The whole of the skin is now to be carefully dissected away, not only from the abdomen and thorax, but from the axillary, inguinal, and cervical regions, and the fore and hind legs as well. It is then pinned flat upon the board so as to keep it as far from the abdomen and thorax as possible, for it is from the skin that the chances of contamination are greatest.

It now becomes necessary to proceed very carefully. All incisions from this time on are to be made only through surfaces that have been sterilized. The sterilization is best accomplished by the use of a broad-bladed table-knife that has been heated in a gas-flame. The blade, made quite hot, is to be held upon the region of the linea alba until the tissues of that region begin to burn; it is then held transversely to this line over about the centre of the abdomen, thus making two sterilized tracks, through which the abdomen may be opened by a crucial incision. The sterilization thus accomplished is, of course, directed only against organisms that may have fallen upon the surface from without, and therefore it need not extend deep down through the tissues. In the same way two burned lines may be made from either extremity of the transverse line up to the top of the thorax.

With hot scissors the central longitudinal incision extending from the point of the sternum to the genitalia is to be made without touching the internal viscera. The abdominal wall must therefore be held up during the operation with sterilized forceps or hooks. The cross-incision is made in the same way. When this is completed an incision through the ribs with a pair of heavy, sterilized scissors is made along the

scorched tracks on either side of the thorax. After this the whole anterior wall of the thorax may easily be lifted up, and by severing the connections with the diaphragm it may be completely removed. When this is done and the abdominal flaps laid back, the contents of both cavities are to be inspected and their condition noted without disturbing them.

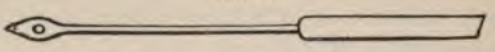
After this the first steps to be taken are to prepare plates or Esmarch tubes from the blood, liver, spleen, kidneys, and any exudates that may exist. This is best done as follows: Heat a scalpel quite hot and apply it to a small surface of the organ from which cultures are to be made. Hold it upon the organ until the surface directly beneath is visibly scorched. Then remove it, heat it again, and while quite hot insert its point through the capsule of the organ. Into the opening thus made insert a sterilized platinum loop, made of wire a little heavier than that commonly employed. Project this deeply into the tissues of the organ; by twisting it about enough material from the centre of the organ can be obtained for making the cultures.

As the resistance offered by the tissue is sometimes too great to permit of puncture with the ordinary wire loop, Nuttall[1] has devised for the purpose a platinum-wire spear which possesses great advantage over the loop. It has the form seen in Fig. 53. It is easily made by beating a piece of heavy platinum wire into a spear-head at one end, and perforating this with a small drill, as seen in the cut. It is attached by the other end to either a metal or glass handle, preferably the former. It can readily be thrust into the densest of the soft tissues,

[1] Centralbatt für Bakteriologie und Parasitenkunde, 1892, Bd. xi. p. 538.

and by twisting it about after its introduction particles of the tissue sufficient for examination are withdrawn in the eye of the spear-head.

FIG. 53.



Nuttall's platinum spear for use at autopsies.

Cultures from the blood are usually made from one of the cavities of the heart, which is always punctured at a point which has been burned in the way given.

In addition to cultures, cover-slips from the site of inoculation, from each organ, and from any exudates that may be present must be made. These, however, are prepared *after* the materials for the cultures have been obtained. They need not be examined immediately, but may be placed aside, under cover, on bits of paper upon which the name of the organ from which they were prepared is written.

When the autopsy is complete and the gross appearances have been carefully noted, small portions of each organ are to be preserved in 95 per cent. alcohol for subsequent examination. Throughout the entire autopsy it must be borne in mind that all cultures, cover-slips, and tissues must be carefully labelled, not only with the name of the organ from which they originate, but with the date, designation of the animal, etc., so that an account of their condition after closer study may be subsequently inserted in the protocol.

The cover-slips are now to be stained, mounted, and

examined microscopically, and the results carefully noted.

The same care with regard to noting, labelling, etc., should be exercised in the subsequent study of the cultures and the hardened tissues, which are to be stained and subjected to microscopic examination. The results of microscopic study of the cover-slip preparations and of those obtained by cultures should in most cases correspond, though it not rarely occurs that bacteria are present in such small numbers in the tissues that their presence may be overlooked microscopically, and still they may appear in the cultures.

If the autopsy has been performed in the proper way, with the precautions given, and sufficiently soon after death, the results of the bacteriological examination should be either negative or the organisms which are isolated should be in pure cultures. This is particularly the case with cultures made from the internal viscera.

Both the cover-slips and cultures made from the point of inoculation are apt to contain a variety of organisms.

If the organism obtained in pure culture from the internal viscera, or those predominating at the point of inoculation of the animal, have caused its death, then subsequent inoculation of pure cultures of this organism into the tissues of a second animal should produce similar results.

When the autopsy is quite finished the remains of the animal should be *burned;* all instruments subjected to either sterilization by steam or boiling for fifteen minutes in a 1 to 2 per cent. soda solution; and the board upon which the animal was tacked, as well as the

tacks, towels, dishes, and all other implements used at the autopsy, be sterilized by steam. All cultures, cover-slips, and, indeed, all articles likely to have infectious material upon them, must be thoroughly sterilized as soon as they are of no further service.

What has been said with regard to the study of dead tissues obtained at autopsy applies equally well to the bacteriological study of tissues and exudates obtained during life. In the latter case, however, certain precautions are always to be observed. In the first place, it is desirable to obtain the materials under aseptic precautions, care being taken that no disinfectant fluids are mixed with them. They should be subjected to study as soon as possible after removal from the body. In the case of tissues that cannot be examined on the spot, they should be placed in a sterile Petri dish or in a stoppered, sterile, wide-mouthed bottle and taken at once to the laboratory. The surface should then be seared with a hot knife and an incision through the seared area into the centre made with a knife that has been sterilized and allowed to cool. From the depths of this incision enough material may be obtained for microscopic examination and for the preparation of cultures. Fluid exudates that must be taken to the laboratory should be collected in either a sterile test-tube, or, better, in a sterile capillary tube that is subsequently sealed at both ends in a gas-flame. When bacteriological examination of the blood during life is required, it is customary to obtain the necessary sample of blood by pricking the skin. It must be remembered, in this connection, that the skin usually contains a number of species of bacteria that

are of no pathological significance and have nothing to do with the disease from which the individual may be suffering. It is manifestly essential to exclude these. It is not possible to exclude them certainly and completely under all circumstances, without a more or less elaborate procedure; but an effort to do so should always be made. As a rule, the greater number of them may be removed from the skin by careful washing with warm water and soap and a sterile brush, after which the skin should be rinsed with alcohol and allowed to dry spontaneously. The drop of blood may then be obtained from the skin thus cleaned by a prick with a sharp, sterilized lancet. The presence in the cultures of a staphylococcus, growing slowly, with white colonies, is a frequent experience, and does not necessarily imply that this organism bears an etiological relation to the disease from which the individual may be suffering (see *staphylococcus epidermidis albus*, page 268).

In the study of many of the common diseases, notably the exanthemata, both at autopsy and during life, by the methods above outlined, the investigation often yields negative results, and yet there is every reason for believing these diseases to be dependent for their existence upon invasion of the body by some form or another of living micro-organisms, capable of growth in the tissues and susceptible of being transmitted from individual to individual, either directly or indirectly. In this connection it is appropriate to call attention to the novel and important technical procedures that have been employed by Nocard and Roux[1] in their investigations conducted in the Pasteur Institute at Paris. In the course of their studies upon the pleuro-

[1] Nocard and Roux: Annales de l'Institut Pasteur, April 25, 1898.

pneumonia of cattle, a disease in which all investigators had hitherto failed to detect by either microscopic or culture-methods any species of bacteria that might reasonably be regarded as etiologically related to the disease, they detected a group of bodies, apparently bacteria, of such infinitesimally small dimensions as entirely to escape detection by the usual methods of examination.

The results of Nocard and Roux were obtained both through the adoption of special methods of cultivation and the use of very high amplifying powers for microscopic examination. The method of cultivation was that suggested in 1896 by Metchnikoff, Roux, and Salimbeni,[1] and is essentially as follows: very thin-walled, small sacs of collodion are sterilized by steam, filled with bouillon, inoculated with the exudate or tissue to be tested, sealed with sterile collodion, and placed in the abdominal cavity of an animal — rabbit, guinea-pig, chicken, dog, sheep, or calf, as the case may require. The wall of the collodion sac is impermeable to bacteria or to leucocytes, but is an osmotic membrane through which fluids and some of their dissolved contents readily diffuse. This diffusion supplies the bacteria within the sacs with such matters from the living fluids of the animal as are apparently essential to their development, while at the same time the bacteria develop uninterruptedly, being protected by the collodion membrane from the antagonistic action of the fixed and wandering cells of the tissues. After a period of from a few days to several months the animal is sacrificed, and the sac removed from the peritoneal cavity and its con-

[1] Metchnikoff, Roux, and Salimbeni: Annales de l'Institut Pasteur, 1896, p. 257.

tents subjected to microscopic examination. This latter part of the work was unsatisfactory when conducted with the usual combinations of lenses employed in bacteriological work. Satisfactory examinations could only be made by the use of very high magnifying powers, about 2000 diameters, and unusually brilliant illumination. When conducted under these conditions the sacs inoculated with matters from the pulmonary exudates of pleuro-pneumonia were found to contain numerous motile points or dots of such extremely small size that it was often impossible to decide as to their exact form. Control-sacs not inoculated, but kept in the peritoneal cavity of the same canal, manifestly under similar conditions, did not reveal the presence of the minute bodies.

In referring at length to this investigation it is not my purpose to discuss the object of it, but only to direct attention to this novel technique, which seems capable of much wider application. There is a group of common maladies, such as measles, scarlet fever, smallpox, etc., of the etiology of which we know nothing, and on which it has hitherto been impossible to shed important light by the usual bacteriological procedures. Nocard and Roux have apparently revealed to us a world of micro-organisms whose existence has hitherto been unsuspected, and it is not unreasonable to suppose that it is among this group that we are to seek for the causative agents of many specific diseases whose etiology is as yet obscure.[1]

It is proper also to call attention to the work of Adami upon progressive portal cirrhosis, in which he too, through the use of very high magnifying lenses,

[1] An excellent review of the paper of Nocard and Roux is to be found in the Philadelphia Medical Journal of June 11, 1898.

has found an infinitesimally small micro-organism in Pictou cattle-disease, "the main feature of which is extensive cirrhosis of the liver accompanied by swelling of the periportal and retroperitoneal lymph-glands. In a certain number of cases of hepatic cirrhosis of human beings Adami further believes that he has found a very minute micro-organism that may possibly stand in causal relation to the process.[1]

[1] Adami: Montreal Medical Journal, July, 1898.

APPLICATION OF THE METHODS OF BACTERIOLOGY.

CHAPTER XIV.

To obtain material with which to begin work.

EXPOSE to the air of an inhabited room a slice of freshly steamed potato or a bit of slightly moistened bread upon a plate for about one hour. Then cover it with an ordinary water-glass, place it in a warm spot (temperature not to exceed that of the human body —37.5° C.), and allow it to remain undisturbed. In from twenty-four to thirty-six hours there will be seen upon the cut surface of the bread or potato small, round, oval, or irregularly round patches which present various appearances. These differences in macroscopic appearance are due in some cases to the presence or absence of color; in others to a higher or lower degree of moisture; in some instances a patch will be glistening and smooth, while its neighbor may be dull and rough or wrinkled; here will appear an island regularly round in outline, and there an area of irregular, ragged deposit. All these gross appearances are of value in aiding us to distinguish between these colonies—for colonies they are, and under the same conditions the organisms composing each of them will always produce growth of exactly the same appearance. It was just such an experiment as this,

accidentally performed, that suggested to Koch a means of separating and isolating in pure cultures the component individuals from mixtures of bacteria, and it was from this observation that the methods of cultivation on solid media were evolved.

If, without molesting these objects, we continue the observations from day to day, we shall notice changes in the colonies, due to the growth and multiplication of the individuals composing them. In some cases the colonies will always retain their sharply cut, round, or oval outline, and will increase but little in size beyond that reached after forty-eight to seventy-two hours; whereas others will spread rapidly and quickly overrun the surface upon which they are growing, and, indeed, grow over the smaller, less rapidly developing colonies. In a number of instances, if the observation be continued long enough, many of these rapidly growing colonies will, after a time, lose their lustrous and smooth or regular surface and will show here and there elevations, which will continue to appear until the whole surface takes on a wrinkled appearance. Again, bubbles may be seen scattered through the colonies. These are due to the escape of gas resulting from fermentation, which the organisms bring about in the medium upon which they are growing. Sometimes peculiar odors due to the same cause will be noticed.

Note carefully all these changes and appearances, as they must be employed subsequently in identifying the individual organisms from which each colony on the medium has developed.

If now we examine these colonies upon the bread or potato with a hand-lens of low magnifying power, we will be enabled to detect differences not noticeable to

the naked eye. In a few cases we may still see nothing more than a smooth, non-characteristic surface; while in others minute, sometimes regularly arranged tiny corrugations may be observed. In one colony they may appear as tolerably regular lines, radiating from a central spot; and again they may appear as concentric rings; and if by the methods which have been described we obtain from these colonies their individual components in pure culture, we shall see that this characteristic arrangement in folds, radii, or concentric rings, or the production of color, is constant under normal conditions.

So much for the simplest naked-eye experiment that can be made in bacteriology, and which serves to furnish the beginner with material upon which to commence his studies. It is not necessary at this time for him to burden his mind with names for these organisms; it is sufficient for him to recognize that they are mostly of different species and that they possess characteristics which will enable him to differentiate the one from the other.

In order now for him to proceed it is necessary that he should have familiarized himself with the methods by which his media are prepared and the means employed in sterilizing them and retaining them sterile—*i. e.*, of preventing the access of foreign germs from without—otherwise his efforts to obtain and retain his organisms as pure cultures will be in vain.

EXPOSURE AND CONTACT.—Make a number of plates from bits of silk used for sutures, after treating them as follows:

Place some of the pieces (about 5 centimetres long) in a sterilized test-tube, and sterilize them by steam

for one hour. At the end of the sterilization remove one piece with sterilized forceps and allow it to brush against your clothing, then make a plate from it; draw another piece across a table and then plate it. Suspend three or four pieces upon a sterilized wire hook and let them hang for twenty minutes free in the air, being sure that they touch nothing but the hook; then plate them separately.

Note the results.

In what way do these experiments differ and how can the differences be explained?

Expose to the air six Petri dishes into which either sterilized gelatin or agar-agar has been poured and allowed to solidify; allow them to remain exposed for five, ten, fifteen, twenty, twenty-five, and thirty minutes in a room where no one is at work. Treat a second set in the same way in a room where several persons are moving about. Be careful that nothing touches them and that they are exposed only to the air. Each dish must be carefully labelled with the time of its exposure.

Do they present different results? What is the reason for this difference?

Which predominate—colonies resulting from the growth of bacteria, or those from common moulds?

How do you account for this condition?

Sprinkle a little fine dust over the surface of a plate of sterile gelatin or agar-agar; examine the dust-particles with the microscope immediately after depositing them on the medium, and again after eighteen to twenty-four hours. What differences do you detect?

CHAPTER XV.

Various experiments in sterilization by steam and by hot air.

PLACE in one of the openings in the cover of the steam sterilizer an accurate thermometer; when the steam has been streaming for a minute or two the thermometer will register 100° C. Wrap in a bundle of towels or rags or pack tightly in cotton a maximum (self-registering) thermometer; let this thermometer be in the centre of a bundle large enough to quite fill the chamber of the sterilizer. At the end of a few minutes' exposure to the streaming steam remove it; it will be found to indicate a temperature of 100° C.

Closer study of the penetration of steam has taught us, however, that the temperature found at the centre of such a mass may sometimes be that of the air in the meshes of the material, and not that of steam, and for this reason the sterilization at that point may not be complete, because hot air at 100° C. has not the sterilizing properties that steam has at the same temperature. It is necessary, therefore, that this air should be expelled from the meshes of the material and its place taken by the steam before sterilization is complete. This is insured by allowing the steam to stream through the substances a few minutes before beginning to calculate the time of exposure. There is as yet no absolutely sure means of saying that the temperature at the centre of the mass is that of hot air or of steam, so that the exact length of time that is required for the expulsion

of the air from the meshes of the material cannot be given.

Determine if the maximum thermometer indicates a temperature of 100° C. at the centre of a moist bundle in the same way as when a dry bundle was employed.

To about 50 c.c. of bouillon add about 1 gramme of chopped hay, and allow it to stand in a warm place for twenty-four hours. At the end of this time it will be found to contain a great variety of organisms. Continue the observation, and ultimately a pellicle will be seen to form on the surface of the fluid. This pellicle is made up of rods which grow as long threads in parallel strands. In many of these rods glistening spores will be seen. After thoroughly shaking, filter the mass through a fine cloth to remove coarser particles.

Pour into each of several test-tubes about 10 c.c. of the filtrate. Allow one tube to remain undisturbed in a warm place. Place another in the steam sterilizer for five minutes; a third for ten minutes; a fourth for one-half hour; a fifth for one hour.

At the end of each of these exposures inoculate a tube of sterilized bouillon from each tube. Likewise make a set of plates or Esmarch tubes upon both gelatin and agar-agar from each tube, and note the results. At the same time prepare a set of plates or Esmarch tubes on agar-agar and on gelatin from the tube which has not been exposed to the action of the steam.

The plates or tubes from the unmolested tube will present colonies of a variety of organisms; separate and study these.

Those from the tube which has been sterilized for

five minutes will present colonies in moderate numbers; but, as a rule, they will represent but a single organism. Study this organism in pure cultures.

The same may be predicted for the tube which has been heated for ten minutes, though the colonies will be fewer in number.

The thirty-minute tube may or may not give one or two colonies of the same organism.

The tube which has been heated for one hour is usually sterile.

The bouillon tubes from the first and second tubes which were heated will usually show the presence of only one organism—the bacillus which gave rise to the pellicle-formation in the original mixture. This organism is *bacillus subtilis*. It is especially adapted to the study of those various degrees of resistance to heat that spore-forming bacteria exhibit at different stages of their development.

Inoculate about 100 c.c. of sterilized bouillon with a very small quantity of a pure culture of this organism, and allow it to stand in a warm place for about six hours. Now subject this culture to the action of steam for five minutes; it will be seen that sterilization, as a rule, is complete.

Treat in the same way a second flask of bouillon, inoculated in the same way with the same organism, but after having stood in a warm place for from forty-eight to seventy-two hours—that is, until spores have formed—and it will be found that sterilization is not complete: the spores of this organism have resisted the action of steam for five minutes.

To determine if sterilization is complete always resort to the culture methods, as the macroscopic and micro-

scopic methods are deceptive; cloudiness of the media or the presence of bacteria microscopically does not always signify that the organisms possess the property of life.

Inoculate in the same way a third flask of bouillon with a *very small* drop from one of the old cultures upon which the pellicle has formed; mix it well and subject it to the action of steam for *two* minutes; then place it to one side for from twenty to twenty-four hours, and again heat for two minutes; allow it to stand for another twenty-four hours, and repeat the process on the third day. No pellicle will be formed, and yet spores were present in the original mixture, and, as we have seen, the spores of this organism are not killed by an exposure of five minutes to steam. How can this result be accounted for?

Saturate several pieces of cotton thread, each about 2 cm. long, in the original decomposed bouillon, and dry them carefully at the ordinary temperature of the room; then at a little higher temperature—about 40° C.—to complete the process. Regulate the temperature of the hot-air sterilizer for about 100° C., and subject several pieces of this infected and dried thread to this temperature for the same lengths of time that we exposed the same organisms in bouillon to the steam, viz., five, ten, thirty, and sixty minutes. At the end of each of these periods remove a bit of thread, and prepare a set of plates or Esmarch tubes from it. Are the results analogous to those obtained when steam was employed?

Increase the temperature of the dry sterilizer and repeat the process. Determine the temperature and time necessary for the destruction of these organisms by dry heat. These threads should not be simply

laid upon the bottom of the sterilizer, but should be suspended from a glass rod, which may be placed inside the oven, extending across its top from side to side.

Place several of the infected threads in the centre of a bundle of rags. Subject this to a temperature necessary to sterilize the threads by the dry method. Treat another similar bundle to sterilization by steam. In what way do the results of the two processes differ?

CHAPTER XVI.

Suppuration—The staphylococcus pyogenes aureus—Staphylococcus pyogenes albus and citreus—Streptococcus pyogenes—Staphylococcus epidermidis albus—Bacillus pyocyaneus—General remarks.

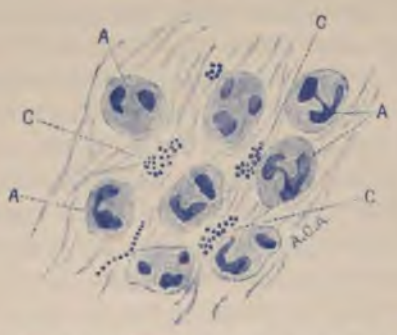
STAPHYLOCOCCUS PYOGENES AUREUS.

PREPARE a set of plates of agar-agar from the pus of an acute abscess or boil that has been opened under antiseptic precautions. Care must be taken that none of the antiseptic used gains access to the culture-tubes, otherwise its antiseptic effect may be operative and the development of the organisms interfered with. It is best, therefore, to take a drop of the pus upon a platinum-wire loop after it has been flowing for a few seconds; even then it must be taken from the mouth of the incision and before it has run over the surface of the skin. At the same time prepare two or three cover-slips from the pus.

Microscopic examination of these slips will reveal the presence of a large number of pus-cells, both multinucleated and with horseshoe-shaped nuclei, some threads of disintegrated and necrotic connective tissue, and, lying here and there throughout the preparation, small round bodies which will sometimes appear singly, sometimes in pairs, and frequently will be seen grouped together somewhat like clusters of grapes. (See Fig. 54.) They stain readily and are commonly located in the material between the pus-cells; very rarely they may be seen in the protoplasmic body of the cell.

(Compare the preparation with a similar one made from the pus of gonorrhea. (See Fig. 57.) In what way do the two preparations differ, the one from the other?)

FIG. 54.



Preparation from pus, showing pus-cells, A, and staphylococci, C.

After twenty-four hours in the incubator the plates will be seen to be studded here and there with yellow or orange-colored colonies, which are usually round, moist, and glistening in their naked-eye appearances. When located in the depths of the medium they are commonly seen to be lozenge or whetstone in shape, while often they appear as irregular stars with blunt points, and again as irregularly lobulated dense masses. In structure they are conspicuous for their density. Under the low objective they appear, when on the surface, as coarsely granular, irregularly round patches, with more or less ragged borders and a dark irregular central mass, which has somewhat the appearance of masses of coarser clumps of the same material as that composing the rest of the colony. Microscopically, these colonies are composed of small round cells, irreg-

ularly grouped together. They are in every way of the same appearance as those seen upon the original cover-slip preparation.

Prepare from one of these colonies a pure stab-culture in gelatin. After thirty-six to forty-eight hours liquefaction of the gelatin along the track of the needle, most conspicuous at its upper end, will be observed. As growth continues the liquefied portion becomes more or less of a stocking-shape, and gradually widens at its upper end into an irregular funnel. This will continue until the whole of the gelatin in the tube eventually becomes fluid. There can always be noticed at the bottom of the liquefying portion an orange-colored or yellow mass composed of a number of the organisms which have sunk to the bottom of the fluid.

On potato the growth is quite luxuriant, appearing as a brilliant, orange-colored layer, somewhat lobulated and a little less moist than when growing upon agar-agar.

It does not produce fermentation with gas-production.

It belongs to the group of facultative aërobes.

In milk it rapidly brings about coagulation with acid reaction.

It is not motile, and being of the family of micrococci does not form endogenous spores. It possesses, however, a marked resistance to detrimental agencies.

In bouillon it causes a diffuse clouding, and after a time presents a yellow sedimentation.

This organism is the commonest of the pathogenic bacteria with which we shall meet. It is *staphylococcus pyogenes aureus*, and is the organism most frequently concerned in the production of acute, circumscribed, suppurative inflammations. It is almost every-

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troduced, or the intestines be mechani-
that there is a disturbance in their cir-
the introduction of these organisms is
llowed by acute and fatal peritonitis. (Hal-

ther hand, the results which follow their in-
into the circulation are practically constant.

The Johns Hopkins Hospital Reports. Report in Sur-
1891, vol. ii. No. 5, pp. 301-303.

If one inject into the circulation of the rabbit through a vein of the ear, or in any other way, from 0.1 to 0.3 c.c. of a bouillon culture or watery suspension of a virulent variety of this organism, a fatal pyæmia always follows in from two and one-half to three days. A few hours before death the animal is frequently seen to have severe convulsions. Now and then excessive secretion of urine is noticed. The animal may appear in moderately good condition until from eight to ten hours before death. At the autopsy a typical picture presents: the voluntary muscles are seen to be marked here and there by yellow spots, which average the size of a flaxseed, and are of about the same shape. They lie usually with their long axis running longitudinally between the muscle-fibres. As the abdominal and thoracic cavities are opened the diaphragm is often seen to be studded with them. Frequently the pericardial sac is distended with a clear gelatinous fluid, and almost constantly the yellow points are seen in the myocardium. The kidneys are rarely without them; here they appear on the surface as isolated yellow points, or, again, are seen as conglomerate masses of small yellow points which occupy, as a rule, the area fed by a single vessel. If one makes a section into one of these yellow points, it will be seen to extend deep down through the substance of the kidney as a yellow, wedge-shaped mass, the base of the wedge being at the surface of the organ.

It is very rare that these abscesses—for abscesses the yellow points are, as we shall see when we come to study them more closely—are found either in the liver, spleen, or brain; their usual location being, as said, in the kidney, myocardium, and voluntary muscles.

These minute abscesses contain a dry, cheesy, necrotic centre, in which the staphylococci are present in large numbers, as may be seen upon cover-slips prepared from them. This organism may also be obtained in pure culture from these suppurating foci.

Preserve in Müller's fluid and in alcohol duplicate bits of all the tissues in which the abscesses are located. When these tissues are hard enough to cut sections should be made through the abscess-points and the histological changes carefully studied.

MICROSCOPIC STUDY OF COVER-SLIPS AND SECTIONS. —In cover-slip preparations this organism stains readily with the ordinary dyes. In tissues, however, it is best to employ some method by means of which contrast-stains may be utilized, and the location and grouping of the organisms in the tissues rendered more conspicuous. When stained, sections of tissues containing these small abscesses present the following appearances:

To the naked eye will be seen here and there in the section, if the abscesses are very numerous, small, darkly stained areas which range in size from that of a pin-point up to those having a diameter of from 1 to 2 mm. These points, when in the kidney, may be round or oval in outline; or may appear wedge-shaped, with the base of the wedge toward the surface of the organ. The differences in shape depend frequently upon the direction in which the section has been made through the kidney. In the muscles they are irregularly round or oval.

When quite small they appear, in stained sections, to the naked eye, as simple, round or oval, darkly stained points; but when they are in a more advanced stage a pale centre can usually be made out.

When magnified they appear in the earliest stages as

minute aggregations of small cells, the nuclei of which stain intensely. Almost always evidences of progressing necrosis can be seen about the centre of these cell-accumulations. The normal structure of the cells of the tissues is more or less destroyed; there is seen a granular condition due to cell-fragmentation; at different points about the centre of this area the tissue appears cloudy and the tissue-cells do not stain readily. Round about and through this spot are seen the nuclei of pus-cells, many of which are undergoing disintegration. In the smallest of these beginning abscesses the staphylococci are to be seen scattered about the centre of the necrotic tissue; but in a more advanced stage they are commonly seen massed together in very large numbers in the form commonly referred to as *emboli of micrococci.*

The localized necrosis of the tissues which is seen at the centre of the abscess is the direct result of the action of a poison produced by the bacteria, and represents the starting-point for all abscess-formations.

When the process is further developed the different parts of the abscess are more easily detected. They then present in sections somewhat the following conditions: at the centre can be seen a dense, granular mass which stains readily with the basic aniline dyes, and when highly magnified is found to be made up of staphylococci. Sometimes the shape of this mass of staphylococci corresponds to that of the capillary in which the organisms became lodged and developed. Immediately about the embolus of cocci the tissues are seen to be in an advanced stage of necrosis. Their structure is almost completely destroyed, though the destruction is seen to be more advanced in some of the

elements of the tissues than in others. As we approach the periphery of this faintly stained necrotic area it becomes marked here and there with granular bodies, irregular in size and shape, which stain in the same way as do the nuclei of the pus-cells and represent the result of disintegration going on in these cells.

Beyond this area we come upon a dense, deeply stained zone, consisting of closely packed pus-cells; of granular detritus resulting from destructive processes acting upon these cells; and of the normal cellular and connective-tissue elements of the part. Here and there through this zone will be seen localized areas of beginning death of the tissues. This zone gradually fades away into the healthy surrounding tissues. It constitutes the so-called "abscess-wall."

Such is the picture presented by the miliary abscess when produced experimentally in the rabbit, and it corresponds from beginning to end with the pathological changes which accompany the formation of larger abscesses in the tissues of human beings.

From these small abscesses in the tissues of the rabbit *staphylococcus pyogenes aureus* may again be obtained in pure culture, and will present identically the same characteristics that were possessed by the culture with which the animal was inoculated.

THE LESS COMMON PYOGENIC ORGANISMS.

The pus of an acute abscess in the human being may sometimes contain other organisms beside *staphylococcus pyogenes aureus*. *Staphylococcus pyogenes albus* and *citreus* may be found. The colonies of the former are white, those of the latter are lemon-color. With these exceptions they are in all essential cultural peculi-

arities similar to *staphylococcus aureus*. As a rule, they are not virulent for animals, and when they do possess pathogenic properties it is in a much lower degree than is commonly the case with the golden staphylococcus. *Streptococcus pyogenes* is also sometimes present. The commonest of the pyogenic organisms, however, is that just described, viz., *staphylococcus pyogenes aureus*. An organism that is almost universally present in the skin, and is often concerned in producing mild forms of inflammation, is *staphylococcus epidermidis albus* (Welch), an organism that may readily be confused with *staphylococcus albus*. It is distinguished from the latter by the slowness with which it liquefies gelatin and by the comparative absence of pathogenic properties when injected into the circulation of rabbits. Welch regards this organism as a variety of *staphylococcus pyogenes albus*.

Streptococcus Pyogenes. — From a spreading phlegmonous inflammation prepare cover-slips and cultures. What is the predominating organism? Does it appear in the form of regular clusters like those of grapes, or have its individuals a definite, regular arrangement? (See Fig. 55.) Are its colonies like those of *staphylococcus pyogenes aureus?*

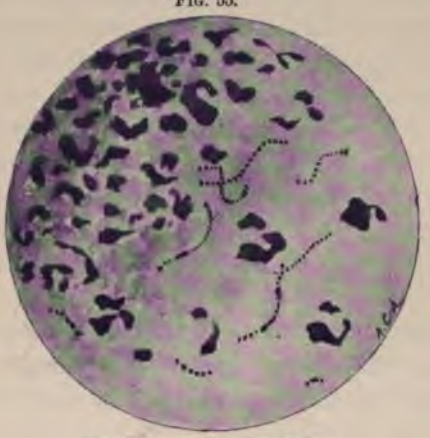
Isolate this organism in pure cultures. In these cultures it will be found on microscopic examination to present an arrangement somewhat like a chain of beads. (Fig. 56.)

Determine its peculiarities and describe them accurately. They should be as follows:

Upon microscopic examination a micrococcus should be found, but differing in its arrangement from the staphylococci just described. The single cells are not

scattered irregularly or arranged in clumps similar to bunches of grapes, but are joined together in chains like strands of beads. These strands are sometimes regular

FIG. 55.



Streptococcus pyogenes in pus.

in the arrangement and size of the individual cells composing them, but more commonly certain irregular groups may be seen in them. Here they appear as if two or

FIG. 56.

Streptococcus pyogenes.

three cells have fused together to form a link, so to speak, in the chain, that is somewhat longer than the remaining links; again, portions of the chain may be thinner

than the rest, or may appear broken or ragged. Commonly the individuals comprising this chain of cocci are not round, but appear flattened on the sides adjacent to one another. The chains are sometimes short, consisting of four to six cells; or again they may be much longer, and extend from a half to two-thirds across the field of the microscope.

Under artificial conditions it sometimes grows well, and can be cultivated through many generations, while at other times it rapidly loses its vitality. When isolated from the diseased area upon artificial media it seems to retain its vitality for a longer period if replanted upon fresh media every day or two for a time; but if the first generation is transplanted and is allowed to remain upon the original medium, it is not uncommon to find the organism incapable of further cultivation after a week or ten days.

Under no conditions is the growth of this organism very luxuriant.

On gelatin plates its colonies appear after forty-eight to seventy-two hours as very small, flat, round points of a bluish-white or opalescent appearance. They do not cause liquefaction of the gelatin, and in size they rarely exceed 0.6–0.8 mm. in diameter. Under low magnifying power they have a brownish or yellowish tinge by transmitted light, and are finely granular. As the colonies become older their regular border may become slightly irregular or notched.

In stab-cultures in gelatin they grow along the entire needle-track as a finely granular line, the granules representing minute colonies of the organism. On the surface the growth does not usually extend beyond the point of puncture.

On agar-agar plates the colonies appear as minute pearly points, which when slightly magnified are seen to be finely granular, of a light-brownish color, and regular at their margins.

When smeared upon the surface of agar-agar or gelatin slants the growth that results is a thin, pearly, finely granular layer, consisting of minute colonies growing closely side by side. Its most luxuriant growth is seen on glycerin-agar-agar at the temperature of the body (37.5° C.), and its least on gelatin at from 18° to 20° C.

On blood-serum its colonies present little that is characteristic; they appear as small, moist, whitish points, from 0.6 to 0.8 mm. in diameter, that are slightly elevated above the surface of the serum. They do not coalesce to form a layer over the surface, but remain as isolated colonies.

On potato no visible development appears, but after a short time (thirty-six to seventy-two hours) there is a slight increase of moisture about the point of inoculation, and microscopic examination shows that multiplication of the organisms placed at this point has occurred.

In milk its conduct is not always the same, some cultures causing a separation of the milk into a firm clot and colorless whey, while others do not produce this coagulation. The latter, when cultivated in milk of a neutral or slightly alkaline reaction, to which a few drops of litmus tincture have been added, produce a very faint pink color after twenty-four hours at 37.5° C.; there is no coagulation.

In bouillon it grows as tangled masses or clumps, which upon microscopic examination are seen to consist of long chains of cocci twisted or matted together.

It grows best at the temperature of the body (37.5° C.), and develops, but less rapidly, at the ordinary room-temperature. When virulent this property is said by Petruschky to be preserved by retaining the cultures in the ice-chest after they have been growing on gelatin for two days at 22° C.

Its virulence may often be increased by passing it through a series of susceptible animals.

It is a facultative anaërobe.

It stains with the ordinary aniline dyes, and is not decolorized when subjected to Gram's method.

It is not motile, and, being a micrococcus, does not form endogenous spores. Under artificial conditions we have no reason to believe that it enters a stage in which its resistance to detrimental agencies is increased. In the tissues of the body, however, it appears to possess marked vitality, for it is not rare to observe recurrences of inflammatory conditions due to this organism, often at a relatively long time after the primary site of infection has healed.

The results of its inoculation into the tissues of lower animals are described by Rosenbach and Passet as protracted, progressive, erysipelatoid inflammations; and Fehleisen, who described a streptococcus in erysipelas that is in all probability identical with the *streptococcus pyogenes* under consideration, stated that it produced in the tissues of rabbits (the base of the ear) a sharply defined, migratory reddening without pus-formation. The writer has encountered a culture of this organism that possessed the property of inducing erysipelas when introduced into the skin of the ear, and disseminated abscess-formation when injected into the circulation of rabbits. This observation has an im-

portant bearing upon the question concerning the identity of streptococci found in various inflammatory conditions, such, for instance, as the spreading erysipelatoid manifestations on the one hand, and the circumscribed abscess-formations on the other.

The results that follow upon the inoculation of animals with cultures of streptococci obtained from various inflammatory lesions are, as a rule, inconstant. At times cultures will be encountered that are apparently without virulence, no matter how tested; while again cultures from other sources exhibit the most marked pathogenic properties, even when employed in almost infinitesimal quantities. Between these extremes every gradation may be expected. The virulence of a culture is not necessarily proportional to the intensity of the pathological process from which it is derived.

There is never any certainty of faithfully reproducing, by inoculation into susceptible animals, the pathological lesion from which a culture of the organism may have been obtained. The introduction into a susceptible animal of a culture derived from either a spreading phlegmon or an erysipelatous inflammation may result in erysipelas, general septicæmia, local abscess-formation, or, as said, may have no effect at all. Cultures may be encountered that are pathogenic for one susceptible species of animals and not for another.

Under the ordinary conditions of artificial cultivation fully virulent varieties of *streptococcus pyogenes* usually lose their virulence after a short time. Petruschky[1] preserves this property by cultivation upon nutrient gelatin for two days at 22° C., keeping

[1] Petruschky: Centralblatt für Bakteriologie und Parasitenkunde, 1895, Abth. i. Band xvii.

the cultures after this time in the refrigerator, and transplanting upon fresh gelatin every five or six days. Marmorek[1] finds the virulence preserved by growing the organism in a mixture of 2 parts of horse or human blood-serum and 1 part of nutrient bouillon, or of 1 part of ascites-fluid and 2 parts of bouillon.

Certain authors are of the opinion that a relation exists between virulence and the length of the chains formed by streptococci when growing in fluid media. It is held that those forming the long chains, *streptococcus longus*, are the only ones concerned in animal pathology, and hence the only ones by which pathogenic powers may be exhibited; while those forming the short chains, *streptococcus brevis*, are not, as a rule, pathogenic, and may often be readily differentiated from the other variety by more or less gross cultural characteristics, such as slow liquefaction of gelatin, visible growth on potato, etc.[2]

Antistreptococcus Serum.—For a time a great deal of interest was created by the announcement of Marmorek that he had succeeded in inducing in animals a state of immunity from streptococcus infection, and that the blood-serum of such animals when injected into other susceptible animals and human beings possessed not only the property of rendering them insusceptible to this particular form of infection, but even exhibited curative powers in cases already infected. This serum was obtained from horses or asses that had been rendered immune by the gradual introduction into

[1] Marmorek: Annales de l'Institut Pasteur, 1895.

[2] V. Lingelsheim: Zeitschrift für Hygiene, 1891, Band x., and 1892, Band xii. Behring: Centralblatt für Bakteriologie und Parasitenkunde, 1892, Band xii.

their tissues of increasing amounts of virulent streptococci.

A great deal has been said both for and against the claims of Marmorek; but at present the bulk of opinion is unfavorable, or at least very conservative. Many competent clinicians look with favor upon the use of this serum in suitable cases—*i. e.*, genuine, uncomplicated streptococcus infections; but at the same time others have failed to demonstrate its usefulness. Koch and Petruschky failed throughout to confirm Marmorek's results. It is difficult to reconcile the fact that a single or even several attacks of erysipelas or of phlegmonous inflammation in the human being afford no protection from subsequent attacks, with the statement of Marmorek that a condition of complete immunity may be established in animals by the repeated induction of mild manifestations of the disease.

Streptococcus pyogenes is the organism most commonly found in rapidly *spreading* suppuration, while *staphylococcus pyogenes aureus* is most frequently found in *circumscribed* abscess-formations; they may also be found together.

NOTE.—If the opportunity presents, obtain cultures from a case of erysipelas. Compare the organism thus obtained with the streptococcus just mentioned. Inoculate rabbits both subcutaneously and into the circulation with about 0.2 c.c. of pure cultures of these organisms in bouillon. Do the results correspond, and do they in any way suggest the results obtained with *staphylococcus pyogenes aureus* when introduced into animals in the same way? Do these streptococci flourish readily on ordinary media?

The organisms that have just been described are commonly known as the "pyogenic cocci" of Ogston, Rosenbach, and Passet, and up to as late as 1885 were believed to be the specific factors concerned in the production of suppurative inflammations. Since that time, however, there has been considerable modification of this view, and while they are still known to be the most common causes of suppuration, they are also known not to be the only causes of this process.

With the more general application of bacteriological methods to the study of the manifold conditions coming under the eye of the physician, the surgeon, and the pathologist, observations are constantly being made that do not accord with the earlier ideas upon the specific relation of the pyogenic cocci to all forms of suppuration. There is an abundance of evidence to justify the opinion that a number of organisms not commonly classed as pyogenic may, under certain circumstances, assume this property. For example:

The bacillus of typhoid fever has been found in pure culture in osteomyelitis of the ribs, in acute purulent otitis media, in abscess of the soft parts, in the pus of empyema, and in localized fibro-peritonitis, either during its course or as a sequel of typhoid fever.

Bacterium coli commune has been found in pure culture in acute peritonitis, in liver-abscess, in purulent inflammation of the gall-bladder and ducts, and in appendicitis; and Welch[1] has found it in pure culture in fifteen different inflammatory conditions.

Micrococcus lanceolatus (pneumococcus) has been found alone in abscess of the soft parts, in purulent

[1] Welch: "Conditions Underlying the Infection of Wounds," American Journal of the Medical Sciences, November, 1891.

infiltration of the tissues about a fracture, in purulent cerebro-spinal meningitis, in suppurative synovitis, in acute pericarditis, and in acute inflammation of the middle ear.

Moreover, many of the less common organisms have been detected in pure cultures in inflammatory conditions with which they were not previously thought to be concerned, and to which they are not usually related etiologically.

In consideration of such evidence as this it is plain that we can no longer adhere rigidly to the opinions formerly held upon the etiology of suppuration, but must subject them to modifications in conformity with this newer evidence. We now know that there exist bacteria other than the "pyogenic cocci," which, though not normally pyogenic, may give rise to tissue-changes indistinguishable from those produced by the ordinary pus-organisms.[1]

GONOCOCCUS. MICROCOCCUS GONORRHŒÆ.

One observes upon microscopic examination of cover-slips prepared from the pus of acute gonorrhœa that many of the pus-cells contain within their protoplasm numerous small, stained bodies that are usually arranged in pairs. Occasionally a cell is seen that contains only one or two pairs of such bodies; again, a cell will be encountered that is packed with them. Occasionally masses of these small bodies will be seen lying free in the pus. (See Fig. 57.) The majority of the pus-cells do not contain them.

[1] For a more detailed discussion of the subject, see "The Factors Concerned in the Production of Suppuration," International Medical Magazine, Philadelphia, May, 1892.

These small, round, or oval bodies are the so-called "gonococci" discovered by Neisser, and more fully studied subsequently by Bumm, to whom we are indebted for much of our knowledge concerning them.

As the name implies, this organism is a micrococcus,

FIG. 57.

Pus of gonorrhœa, showing diplococci in the bodies of the pus-cells.

and as it is commonly arranged in pairs (flattened at the surfaces in juxtaposition) it is often designated as diplococcus of gonorrhœa. It is always to be found in gonorrhœal pus, and often persists in the urethral discharges and secretions far into the stage of convalescence. It is not present in inflammatory conditions other than those of gonorrhœal origin.

It is easily detected microscopically in the secretions of acute gonorrhœa. In secondary lesions and in very old, chronic cases it is difficult of detection and frequently eludes all efforts to find it. It is stained by the ordinary methods, but perhaps most satisfactorily with the alkaline solution of methylene-blue. Most important as a differential test is its *failure* to stain by the method of Gram. (How does this compare with the

behavior of the other pyogenic cocci when treated in the same way?)

It does not grow upon ordinary nutrient media, and has only been isolated in culture through the employment of special methods. Its growth under artificial conditions seems to depend upon some particular nutrient substance that is supplied by blood or blood-serum, and in all the media that have been successfully used for its cultivation this substance is apparently an essential constituent. By many investigators it is thought that only *human* blood possesses this important ingredient, though this opinion is not universal.[1]

It was first isolated in culture by Bumm, who used for this purpose coagulated human blood-serum obtained from the placenta.

Wertheim improved the method of Bumm by using a mixture of equal parts of sterile human blood-serum and ordinary sterilized nutrient agar-agar, the latter having been liquefied and kept at 50° C. until after the mixture was made, when it was allowed to cool and solidify.

Other investigators have substituted for human blood-serum certain pathological fluids from the human body, such as ascites-fluid, fluid from ovarian cysts, and serous effusions from the pleura and from the joint-cavities.

The method used by Pfeiffer for the cultivation of the bacillus of influenza is also said to have been successfully employed. Abel recommends a needle-prick in the finger as a most convenient source from which to

[1] An instructive article on this subject is that by Foulerton: "On Micrococcus Gonorrhœæ and Gonorrhœal Infection," Transactions of the Bristol Institute of Preventive Medicine, 1897, series i.

obtain the necessary amount of human blood that is to be smeared over the surface of the slanting agar-agar when Pfeiffer's method is employed.

Wright's modification of Steinschneider's method has given such satisfactory results in his hands that it will be given here somewhat in detail. The medium consists of a mixture of urine, blood-serum (human or bovine, either serving the purpose), and nutrient agar-agar. The urine and blood-serum are collected without special aseptic precautions, and subsequently freed from bacteria by filtration through unglazed porcelain. Frequently this is the tedious part of the process, as the serum and urine pass very slowly through the porcelain filters generally employed in laboratories. Wright recommends a filtering cylinder manufactured by the Boston Filter Company as an apparatus that not only gives a sterile filtrate, but also permits of very rapid passage of the fluid.

The details of the method as given by Wright are as follows: "A litre of nutrient agar is prepared in the usual manner, and after filtration it is evaporated to about 600 c.c. This concentration is desirable, so that after dilution with the urine and serum the medium may be sufficiently firm. This concentrated agar is then run into test-tubes and the whole sterilized by steam on three successive days. The quantity of agar placed in each tube is smaller than is usual; this is in order to allow for the subsequent addition of the urine and serum.

"The blood-serum, which need not be free from corpuscles, is first passed through white sand, which is supported in a funnel by filter-paper, in order to remove as far as is possible any particles in suspension,

and is then mixed with half its volume of fresh urine. The mixture of urine and blood-serum is next filtered by suction through an unglazed porcelain cylinder into a receiving-flask, such as chemists use for similar purposes, by means of a water-vacuum pump. This frees the mixture from bacteria.

"The usual precautions are, of course, taken to prevent the contamination of the filtrate, such as the previous sterilization by steam of the cylinder and receiving-flask, besides others which will occur to any bacteriologist.

"To the agar in each test-tube, which is fluid and of a temperature of about 40° C., there is added about one-third to one-half its volume of the filtered mixture of urine and blood-serum. This is conveniently accomplished by pouring the mixture from the receiving-flask through the lateral tube, inserted near its neck directly into the tubes. The preliminary melting of the agar is best effected in the steam sterilizer, in order that any organisms which have found lodgement in the cotton plugs of the tubes may be destroyed. When the agar is melted it is cooled and kept fluid by placing the tubes in a water-bath at 40° C. Each tube, after the addition of the urine and serum to the fluid agar, is quickly shaken to insure a uniform mixture, and is then placed in an inclined position to allow the agar to solidify with a slanting surface. When the medium in the tubes has solidified the tubes are placed in the incubator for about twenty-four hours to test for contaminations, after which they are ready for use."

The successive dilutions are now to be made upon the slanting surface of this mixture, as the mass in the tubes cannot be redissolved without exposure to a de-

gree of heat that apparently interferes with the nutritive value of the serum contained in the medium.

When inoculated with gonorrhœal pus, by smearing a loopful over the surface, the tubes are to be kept at from 37° to 38° C. The organism does not develop properly at a temperature below this point.

After twenty-four hours the colonies of the gonococcus appear on the surface of the medium, according to Wright, as very tiny, grayish, semi-translucent points. After forty-eight hours they may be about 1 millimetre or so in diameter, slightly elevated, with a rounded outline, grayish in color, and semi-translucent by transmitted light. By reflected light their surface has the appearance of frosted glass. Later, if few in number, so that their growth is unimpeded, the colonies may attain a diameter of 2 millimetres or more, become thicker and denser, with a faintly brownish tinge about their centres, and a slightly irregular outline.

Under a low power of the microscope a fully developed colony is seen to consist of a general circular expansion, with thin, translucent, smooth, sharply defined margin, but becoming brownish, granular, and thicker toward the central portion, which is made up of coarse, granular, brown-colored clumps closely packed together.

The appearances coincide with the figure of such a colony given by Wertheim.[1]

Wassermann[2] calls attention to the success he has had in cultivating this organism upon a mixture of swine-serum and nitrose, the latter being a com-

[1] Deutsche med. Wochenschrift, 1891, No. 50; Centralblatt für Gynäkologie, 1891, No. 24.

[2] Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xvii. p. 298.

mercial product chemically known as casein-sodium phosphate.

The preparation of the medium and its composition are as follows:

In an Erlenmeyer flask mix 15 c.c. of swine-serum, as free as possible from hæmoglobin; 30 to 35 c.c. of water; 2 to 3 c.c. of glycerin; and finally 0.8 to 0.9 gramme (*i. e.*, about 2 per cent.) of nitrose. This is boiled, with gentle agitation, over a free flame, until all ingredients are dissolved and the cloudy fluid has become quite clear. After such boiling the mixture can be sterilized by steam without precipitating the albumin, and may then be kept indefinitely ready for use.

When needed, the flask and its contents are heated to 50° C.; from six to eight tubes of 2 per cent. peptone-agar-agar are dissolved by boiling, brought to 50° C., and then mixed with the solution in the flask and the mass poured into Petri dishes. Upon the surface of this serum-nitrose-agar the cultivation is to be conducted. Wassermann lays particular stress upon two points that are essential to success, viz., the preliminary boiling of the serum-nitrose mixture *before* steam sterilization, as this prevents precipitation of the albumin; and the necessity of having both the serum-nitrose mixture and the agar-agar, to be mixed with it, at not over 50° C., for if they are at boiling temperature when mixed, or if they are brought to the boiling temperature *after* mixing, the albumin will be precipitated notwithstanding the presence of the nitrose, which otherwise prevents this.

Wassermann further observes that some samples of serum require to be more highly diluted with water than in the proportions given above; that the agar-agar should be feebly, but distinctly, alkaline to litmus,

causing no reddening whatever of blue litmus paper; and, finally, that the Petri dishes containing the solidified medium on which the cultures are growing are best kept bottom upward, so as to prevent water of condensation collecting on the surface. By the use of the above medium he has cultivated the gonococcus from about one hundred different cases.

If transplanted from the original culture to either glycerin-agar-agar or to Löffler's serum-mixture, a growth is sometimes observed, more often in the latter than in the former, but of so feeble a nature that these substances cannot be regarded as suitable for its cultivation. As a rule, development does not occur on glycerin-agar.

Microscopic examination of colonies of this organism reveals the presence of a diplococcus somewhat larger than the ordinary pyogenic cocci. The opposed surfaces of the individual cells that comprise the couplets are flattened and separated by a narrow slit. At times the cocci are arranged as tetrads.

This organism cannot be grown at a temperature lower than that of the human body, and cultures that have been obtained by either of the favorable methods are said to lose their vitality when kept at ordinary room-temperature for about two days.

It is killed in a few hours by drying.

Cultures retain their vitality under favorable conditions of nutrition, temperature, and moisture for from three to four weeks.

This organism is without pathogenic properties for monkeys, dogs, and horses, as well as for the ordinary smaller animals used for this purpose in the laboratory.

In man typical gonorrhœa has been produced on several occasions by the introduction into the urethra of pure cultures of this organism.

In addition to its causal relation to specific urethritis, it is the cause of gonorrhœal prostatitis in man, of gonorrhœal proctitis in both sexes, and of gonorrhœal inflammation of the urethra, of Bartholin's glands, of the cervix uteri, and of the vagina in women and young girls. It is etiologically related to the specific conjunctivitis (ophthalmia neonatorum) of young infants, and also occasionally to ophthalmia in adults.

Secondarily, it is concerned in specific inflammations of the tubes and ovaries, of the lymphatics communicating with the genitalia, of the serous surfaces of joints, and of those of the heart, lungs, and abdominal cavity.

Other species of micrococci have from time to time been described as occurring in the pus of acute urethritis and of other purulent inflammations. Many of these are of no significance. Some of them possess peculiarities that might lead to confusion. The diplococcus described by Heiman[1] has certain points of resemblance to the gonococcus, such as its location in the bodies of pus-cells, its grouping as diplococci, its size and general appearance; but it is still readily distinguished from the gonococcus by its retention of color when stained by Gram's method. The diplococcus detected by Bumm in puerperal cystitis is likewise often found within pus-cells; but it is readily differentiated from the gonococcus by its growth upon ordinary nutrient media. *Diplococcus intracellularis meningitidis* of Weichselbaum, isolated from the pus of cerebro-spinal meningitis, is microscopically also strikingly like the gono-

[1] New York Medical Record, June 22, 1895.

coccus as it is seen in pus; but, unlike the latter organism, may easily be cultivated by the ordinary culture-methods.

Positive and Negative Distinguishing Peculiarities of the Gonococcus.—Since gonorrhœal discharges may be contaminated with pyogenic cocci other than those causing the specific inflammation, it is important in efforts to isolate this organism that the differential tests be borne in mind and put into practice. The gonococcus is differentiated from the commoner pyogenic organisms by the following peculiarities:

First, it is practically always seen in the form of diplococci, the pair of individual cells having the appearance of two hemispheres, with the diameters opposed and separated from one another by a narrow, colorless slit. (Is this the case with *staphylococcus* or *streptococcus pyogenes?*)

Second, in gonorrhœal pus it is practically always to be found *within* the protoplasmic bodies of pus-cells. (How does this compare with the conditions found in ordinary pus?)

Third, it stains readily with the ordinary staining-reagents, *but loses its color when treated by the method of Gram.* (Treat a cover-slip from ordinary pus by this method and note the result.)

Fourth, it does not develop upon any of the ordinary media used in the laboratory; while the common pus-organisms, with perhaps the exception of the streptococci, are vigorous growers and are not markedly fastidious as to their nutritive medium.

Fifth, when obtained in pure culture by either of the special procedures noted above, its cultivation may be continued upon the same medium; but growth will

usually not be observed if it is transplanted to ordinary nutrient gelatin, agar-agar, bouillon, or potato; should it grow under these circumstances its development will be very feeble. (Is this the case with common pus-producers?)

Sixth, it has no pathogenic properties for animals, while several of the pyogenic cocci, notably *staphylococcus aureus* and *streptococcus pyogenes*, are usually capable of exciting pathological conditions. (This is less commonly true of *streptococcus pyogenes* than of *staphylococcus aureus*.)

BACILLUS PYOCYANEUS (BACILLUS OF GREEN PUS).

Another common organism that may properly be mentioned at this place, though perhaps not strictly pyogenic, is a bacillus frequently found in discharges from wounds, viz., *bacillus pyocyaneus*, or bacillus of green pus, or of blue pus, or of blue-green pus, as it is commonly called. *Bacillus pyocyaneus* is a delicate rod with rounded or pointed ends. It is actively motile; does not form spores. As seen in preparations made from cultures it is commonly clustered in irregular masses. It does not form long filaments, there being rarely more than four joined end to end, and most frequently occurs as single cells.

It grows readily on all artificial media, and gives to some of them a bright-green color that is most conspicuous where it is in contact with the air. This green color, which becomes more and more marked as growth advances, is not seen in the growth itself to any extent, but is diffused through the medium on which the organism is developing. Ultimately this color becomes much darker, and in very old agar-agar cultures may become

almost black (sometimes very dark-blue green, at others brownish-black).

Its growth on gelatin in stab-cultures is accompanied by liquefaction and the diffusion of a bright-green color throughout the surrounding unliquefied medium. As liquefaction continues, and the whole of the gelatin ultimately becomes fluid, the green color is confined

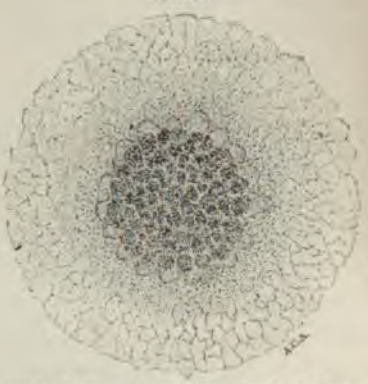
FIG. 58.

Stab-culture of *b. pyocyaneus* in gelatin after twenty-eight hours at 22°C.

FIG. 59.

Colony of *b. pyocyaneus* after twenty-four hours on gelatin at 20°–22° C.

FIG. 60.



Colony of *b. pyocyaneus* after forty-two hours on gelatin at 20°–22° C.

to the superficial layers in contact with the air. The form taken by the liquefying portion of the gelatin in the earliest stages of development is somewhat that of an irregular, slender funnel. (See Fig. 58.)

On gelatin plates the colonies develop rapidly; they

are not sharply circumscribed, but usually present at first a fringe of delicate filaments about their periphery. (See Fig. 59.) As growth progresses and liquefaction becomes more advanced the central mass of the colony sinks into the liquid, while at the same time there is an extension of the colony laterally. At this stage the colony, when slightly magnified, may present various appearances, the most common being that shown in Fig. 60.

The gelatin between the growing colonies takes on a bright yellowish-green color; but as growth is comparatively rapid, it is quickly entirely liquefied, and one often sees the colonies floating about in the pale-green fluid.

On agar-agar the growth is dry, sometimes with a slight metallic lustre, and is of a whitish or greenish-white color, while the surrounding agar-agar is bright green. With time this bright green becomes darker, passing into blue-green, and finally turns almost black.

On potato the growth is brownish, dry, and slightly elevated above the surface. In some cultures the potato about the line of growth becomes green; in others this change is not so noticeable. With many cultures a peculiar phenomenon, consisting of a change of color from brown to green, may be produced by lightly touching the growth with a sterile platinum needle. The change occurs only at the point touched. It is best seen in cultures that have been kept in the incubator for from seventy-two to ninety-six hours. It occurs in from one to three minutes after touching with the needle, and may last from ten minutes to half an hour. This is the "chameleon phenomenon" of Paul Ernst.

In bouillon the green color appears, and the growth is seen in the form of delicate flocculi. A very delicate mycoderma is also produced.

In milk it causes an acid reaction, with coincident coagulation of the casein.

On blood-serum and egg-albumin its growth is accompanied by liquefaction. The growth on coagulated egg-albumin is seen as a dirty-gray deposit surrounded by a narrow brownish zone; the remaining portion of the medium is bright green in color. As the culture becomes older the green may give way to a brown discoloration.

In peptone solution (double strength) it causes a bluish-green color. In one of four cultures from different sources we observed the production of a distinct blue color.

It produces indol.

It stains with the ordinary dyes, and its flagella may be readily demonstrated by Löffler's method of staining.

Inoculation into Animals.—As a rule, cultures of this organism obtained directly from the discharges of the wound are capable, when introduced into animals, of producing diseased conditions; but cultures kept on artificial media for a long time may in part, or completely, lose this power.

When guinea-pigs or rabbits are inoculated subcutaneously with 1 c.c. of virulent fluid cultures of this organism, death usually results in from eighteen to thirty-six hours. At the seat of inoculation there are found an extensive purulent infiltration of the tissues and a marked zone of inflammatory œdema.

When introduced directly into the peritoneal cavity

the results are also fatal, and at autopsy a genuine fibrinous peritonitis is found. There is usually an accumulation of serum in both the peritoneal and pleural cavities. At autopsies after both methods of inoculation the organisms will be found in pure cultures in the blood and internal viscera.

When animals are inoculated with small doses (less than 1 c.c. of a bouillon culture) of this organism death may not ensue, and only a local inflammatory reaction (abscess-formation) may be set up. In these cases the animals are usually protected against subsequent inoculation with doses that would otherwise prove fatal.

Most interesting in connection with *bacillus pyocyaneus* is the fact, as brought out in the experiments of Bouchard, and of Charrin and others, that its products possess the power of counteracting the pathogenic activities of *bacillus anthracis*. That is to say, if an animal be inoculated with a virulent anthrax culture, and soon after be inoculated with a culture of *bacillus pyocyaneus*, the fatal effects of the former inoculation may be prevented. Emmerich and Löw[1] are inclined to attribute this to the direct bacteriolytic action of the pyocyaneus *enzymes* upon the anthrax bacilli introduced into the tissues.

In the literature upon the green-producing organisms that have been found in inflammatory conditions several varieties—believed to be distinct species—have been described; but when cultivated side by side their biological differences are seen to be so slight as to render it probable that they are but modifications of one and the same species.

[1] Münchener med. Wochenschrift, 1898, No. 40; Centralblatt für Bakteriologie und Parasitenkunde, 1899, Abt. i. No. 1, p. 33.

THE BACILLUS OF BUBONIC PLAGUE.

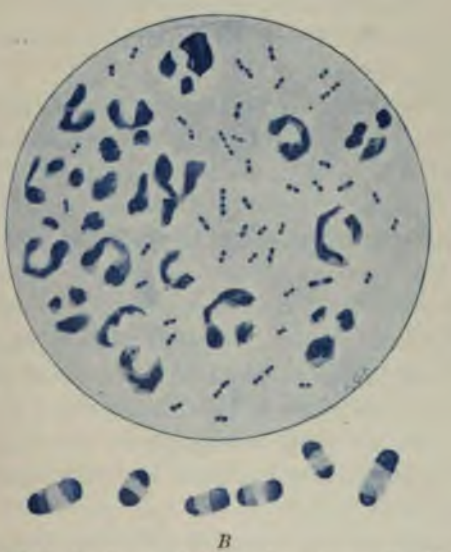
Before passing from the subject of suppuration it may not be inappropriate to call attention to the light that modern methods of investigation have shed upon the etiology of bubonic plague, an epidemic disease characterized by suppuration of the lymphatic glands, and accompanied by a very high rate of mortality.

This pestilence, probably endemic in certain sections of the Orient, is one of the most conspicuous epidemic diseases of history. Since early in the Christian era epidemics and pandemics of plague have made their appearance in Europe at different times. During and for a time after the Middle Ages it was more or less frequent in India, China, Arabia, Northern Africa, Italy, France, Germany, and Great Britain. In history it is variously known as the "Justinian Plague" of the sixth century, the "Black Death" of the fourteenth century, and the "Great Plague of London" of the seventeenth century, though it is difficult to say to what extent these pestilences were uncomplicated manifestations of genuine bubonic plague. During the existence of the Justinian Plague 10,000 people are said to have died in Constantinople in a single day, and Hecker estimates that during the pandemic of the Black Death 25,000,000 people (a quarter of the entire population of Europe) succumbed to the disease. During the Great Plague of London (1664–'65) the total mortality for one year was 68,596, out of an estimated population of 460,000 souls.

It is not surprising to learn that it was to guard against the plague that quarantine regulations were first established.

The first and certainly the most exact information up to date concerning the cause and pathology of the plague resulted from the investigations of Yersin, of Kitasato, and of Aoyama, conducted during the epidemic of 1894 in Hong-Kong, China; though since then numerous other investigators have made addi-

FIG. 61.



Bacillus of bubonic plague: *A*, in pus from suppurating bubo; *B*, the bacillus very much enlarged to show peculiar polar staining.

tional important contributions to our knowledge of the subject. The results of these studies indicate that bubonic plague is an infectious, not markedly contagious, disease that depends for its existence upon the presence in the tissues of a specific micro-organism—the so-called plague or pest bacillus.

This organism is described as a short, oval bacillus, usually seen single, sometimes joined end to end in pairs or threes, less commonly as longer threads. It stains more readily at its ends than at its centre. It is sometimes capsulated; is non-spore-forming; is aërobic, and is non-motile. It is found in large numbers in suppurating glands, and in much smaller numbers in the circulating blood. (Fig. 61.)

It is demonstrable in cover-slip preparations made from the pus and in sections of the glands by the ordinary staining-methods.

Wilm [1] has found it by culture methods in the spleen, lungs, liver, kidneys, stomach, walls of the intestine, urine, and intestinal contents of fresh cadavers; and during life in the blood, expectoration, fæces, and urine of patients sick of plague. He failed to find it in the perspiration.

Yersin states that it does not retain the color when treated by the method of Gram; while Kitasato says that it at one time stains by this method and at another it becomes decolorized. Aoyama observed that those bacilli within the suppurating glands were decolorized, while those in the blood retained the stain when treated by Gram's method.

Since there is often a mixed infection in these cases, it appears likely that the above discrepancy may be attributed to individual peculiarities of different species of bacteria that were under examination, an opinion that is borne out by more recent studies, from which it has been decided that the genuine plague or pest-bacillus does not stain by Gram's method.

It may be cultivated upon ordinary nutrient media,

[1] Wilm: Hygienische Rundschau, 1897, p. 217.

though preference is given to a neutral or slightly alkaline 2 per cent. peptone solution containing from 1 to 2 per cent. of gelatin.

The most favorable temperature for its growth is between 36° and 39° C. Its colonies on glycerin-agar-agar and on coagulated blood-serum are described as iridescent, transparent, and whitish. On gelatin at 18°–20° C. it develops as small, sharply defined, white colonies without liquefaction of the medium. In stab-cultures it develops both on the surface and along the track of the needle. Its growth is slow. It does not cause a diffuse clouding of bouillon, but grows rather as irregular, flocculent clumps that adhere to the sides or sink to the bottom of the vessel, leaving the fluid clear. It shows but limited growth on potato. It does not ferment glucose with production of gas, nor does it form indol. It coagulates milk.

This organism is killed by drying at ordinary room-temperature in four days. It is killed in three to four hours by direct sunlight. It is destroyed in a half hour by 80° C., and in a few minutes by 100° C. (steam). It is killed in one hour by 1 per cent. carbolic acid and in two hours by 1 per cent. milk of lime.

It is pathogenic for rats, mice, guinea-pigs, rabbits, hogs, horses, monkeys, cats, chickens, and sparrows. Pigeons, hedgehogs, and frogs are immune, and dogs and bovines are apparently so.[1] Animals succumb to subcutaneous inoculation in from two to three days. According to Yersin, the site of subcutaneous inoculation becomes œdematous and the neighboring lymphatics are enlarged in a few hours. After twenty-four

[1] Nuttall: Centralblatt für Bakteriologie und Parasitenkunde, 1897, Abt. i., Band xxii. p. 97.

and it is said that from slowly developing, chronic buboes non-virulent or feebly virulent cultures are often obtained. Variations in the degree of virulence have been observed in different colonies from the same source. Virulence is said by Yersin, Calmette, and Borrel[1] to be accentuated by passing the organism through a series of susceptible animals.

In man the bacilli are most numerous in the enlarged, suppurating lymphatics. They are present, but in smaller numbers, in the blood and the internal organs.

It has been observed that in the suppurating lymphatic glands of man a variety of organisms may be present, but among them are always the plague bacillus. Occasionally, micrococci predominate. In these cases of mixed infection the pest bacilli are said to stain less intensely with alkaline methylene-blue than do the streptococci, and more intensely than do the staphylococci that are present. Also, in this event, the streptococci retain the Gram stain, while the pest bacilli do not and the staphylococci may or may not. It has been suggested that possibly the organisms found by Kitasato in the blood, and which he describes as pest bacilli, that retained the color when treated by the method of Gram, were pairs of micrococci, and not bacilli at all.

It is the opinion of Aoyama that the suppuration of the glands is not caused by the plague bacillus, but is rather the result of the action of the pyogenic cocci with which it is so often associated. It is also his belief that the most important and frequent mode of infection in man is through wounds of the skin. He does not regard either the air-passages or the alimentary

[1] Annales de l'Institut Pasteur, 1895, p. 589.

tract as frequent portals of infection. Wilm, on the contrary, is inclined to regard the alimentary tract as a frequent portal of infection.[1]

The order in which the lymphatics manifest disease appears to depend upon the location of the primary infection. That is to say, if it is upon the feet, as of persons who go barefooted, the superficial and deep inguinal glands are the first to show signs of the disease; while if infection occurs through wounds of the hand, the buboes appear first in the axillary region. As a rule, the wound through which infection is received shows little or no inflammatory reaction.[2]

Wyssokowitz and Zabolotny[3] call attention to the fact that the blood of patients convalescing from plague has an agglutinating action upon fluid cultures of the plague bacillus analogous to that observed when the blood-serum of typhoid or of cholera patients is mixed with similar cultures of the typhoid or the cholera bacillus.

Yersin, Calmette, and Borrel[4] have demonstrated that the general principles underlying the establishment of artificial immunity apply as well to this disease as to a number of others. They have shown that by the use of dead cultures (destroyed by heat) of the plague bacillus animals may be rendered immune from infection by the virulent living organism. They have also shown that the serum of the blood of these animals is

[1] Wilm: loc. cit.

[2] The works of Yersin, of Kitasato, and of Aoyama have been exhaustively reviewed by Flexner in the Bulletin of the Johns Hopkins Hospital, 1894, vol. v. p. 96, and 1896, vol. vii. p. 180. I am indebted to these reviews for much that is here presented on this subject.

[3] Annales de l'Institut Pasteur, 1897, p. 663.

[4] Ebenda, 1895, p. 589.

not only capable of conferring immunity upon other animals into which it is injected, but it has curative properties as well, providing it be employed at an early stage of the disease. In 1896 Yersin[1] used the serum of artificially immunized horses in the treatment of plague in human beings. Of 26 persons (3 in Canton and 23 in Amoy, China) who received injections of the serum during the early stages of the disease, in no case later than the fifth day, only 2 died. Comparing this mortality of 7.6 per cent. with the mortality of 80 per cent. among persons in this epidemic who were treated in other ways, he feels justified in regarding the method as worthy of consideration.

[1] Ebenda, 1897, p. 81.

CHAPTER XVII.

Sputum septicæmia—Septicæmia resulting from the presence of *micrococcus tetragenus* in the tissues.

OBTAIN from a tuberculous patient a sample of fresh sputum—that of the morning is preferable. Spread it in a thin layer upon a black glass plate and select one of the small, white, cheesy masses or dense mucous clumps scattered through it. With a pointed forceps smear this carefully upon two or three thin cover-slips, dry and fix them in the way given for ordinary cover-slip preparations. Stain one in the ordinary way with Löffler's alkaline methylene-blue solution, one other by the Gram method, and a third after the method given for tubercle bacilli in fluids or sputum.

In that stained by Löffler's method—slip No. 1—will be seen a great variety of organisms—round cells, ovals, short and long rods, perhaps spiral forms. But not infrequently will be seen diplococci having more or less of a lancet shape, joined together by their broad ends, the points of the lancet being away from the point of juncture of the two cells. There may also be seen masses of cocci which are conspicuous by their arrangement into groups of fours, the adjacent surfaces being somewhat flattened. They are not sarcina, as one can see by the absence of the division in the third direction of space—they divide in only two directions.

In the slip stained by the Gram method the same groups of cocci which grow as threes and fours will be seen; but the lancet-shaped diplococci now present an altered appearance—they are usually surrounded by a capsule. This capsule is very delicate in structure, and, though a frequent accompaniment, is not constant. It can sometimes be demonstrated by the ordinary methods of staining, though the method of Gram is most satisfactory. (Fig. 63.)

In the third slip, which has been stained by the method given for tubercle bacilli in sputum, if decolorization has been properly conducted and no contrast-stain has been employed, the field will be colorless or of only a very pale rose color. None of the numerous organisms seen in the first slip can now be detected; but instead there will be seen scattered through the field very delicate stained rods, which present, in most instances, a conspicuous beading of their protoplasm—that is, the staining is not homogeneous, but at tolerably regular intervals along each rod are seen alternating stained and unstained points. These rods may be found singly, in groups of twos and threes, and sometimes in clumps consisting of large numbers. When in twos or threes it is not uncommon to find them describing an X or a V in their mode of arrangement, or again they may be seen lying parallel the one to the other.

If contrast-stains are used, these rods will be detected and recognized by their retaining the original color with which they have been stained; whereas all other bacteria in the preparation, as well as the tissue-cells which are in the sputum, will take up the contrast-color. (Fig. 62.)

This delicate beaded rod is *bacillus tuberculosis.* The lancet-shaped diplococcus with the capsule is *micrococcus*

FIG. 62.

Tuberculous sputum stained by Gabbett's method. Tubercle bacilli seen as red rods; all else is stained blue.

lanceolatus. The cocci grouped in fours are *micrococcus tetragenus.*

INOCULATION EXPERIMENT.—Inoculate into the subcutaneous tissues of a guinea-pig one of the small, white, caseous masses similar to that which has been examined microscopically. If death ensues, it will, in all probability, be the result of one of the three following forms of infection:

a. Septicæmia[1] resulting from the introduction into the tissues of an organism frequently present in the sputum. It is designated variously as micrococcus of sputum septicæmia, *diplococcus pneumoniæ,* pneumococcus of Fränkel, meningococcus, *streptococcus lanceolatus Pasteuri, micrococcus lanceolatus, micrococcus Pasteuri, coccus lanceolatus, bacillus salivarius septicus,*

[1] Septicæmia is that form of infection in which the blood is the chief field of activity of the organisms.

bacillus septicus sputigenus, diplococcus lanceolatus capsulatus, micrococcus pneumoniæ crouposæ.

b. A form of septicæmia resulting from invasion of the tissues by an organism frequently seen in the sputum of tuberculous subjects. It is characterized by its tendency to divide into fours. It is *micrococcus tetragenus.*

c. Local or general tuberculosis.

a. SPUTUM SEPTICÆMIA.

If at the end of twenty-four to thirty-six hours the animal be found dead, we may reasonably predict that the result was produced by the introduction into the tissues of the organism of sputum septicæmia above mentioned, viz., *micrococcus lanceolatus,* which is not uncommonly found in the mouths of healthy individuals as well as in other conditions.

Inspection of the seat of inoculation usually reveals a local reaction. "This may be of a serous, fibrinous, hemorrhagic, necrotic, or purulent character. Frequently we may find combinations of these conditions, such as fibro-purulent, fibrino-serous, or sero-hemorrhagic."[1] The most conspicuous naked-eye change undergone by the internal organs will be enlargement of the spleen. It is usually swollen, but may at times be normal in appearance. It is sometimes hard, dark red, and dry; or it may be soft and rich in blood. Frequently there is a limited fibrinous exudation over portions of the peritoneum.

Except in the exudations, the organisms are found only in the lumen of the bloodvessels, where they are

[1] Welch: Johns Hopkins Hospital Bulletin, December, 1892, vol. iii. No. 27.

usually present in enormous numbers. In the blood they are practically always free, and are but rarely found within the bodies of leucocytes.

In stained preparations from the blood and exudates a capsule is not infrequently seen surrounding the organisms. (Fig. 63.) This, however, is not constant.

FIG. 63.

Micrococcus lanceolatus in blood of rabbit. Stained by method of Gram. Decolorization not complete.

If a drop of blood from the dead animal be introduced into the tissues of a second animal (mouse or rabbit), identically the same conditions will be reproduced.

If the organism be isolated in pure culture from the blood of the animal, and a portion of this culture be introduced into the tissues of a susceptible animal, we shall see again the same pathological picture.

It must be remembered, however, that this organism when cultivated for a time on artificial media rapidly loses its pathogenic properties. If, therefore, failure to reproduce the disease after inoculation with old cultures should occur, it is in all probability due to a loss of virulence of the organism.

This organism was discovered by Sternberg in 1880. It was subsequently described by A. Fränkel as the etiological factor in the production of acute fibrinous pneumonia.

It is not uncommonly present in the saliva of healthy individuals, having been found by Sternberg in the oral cavities of about 20 per cent. of healthy persons examined by him, and certain authors are of the opinion that it occurs in the oral or nasal cavities of *all* individuals at various times during life. It is constantly to be detected in the rusty sputum of patients suffering from acute fibrinous pneumonia. Its presence has been detected in the middle ear, in the pericardial sac, in the pleura, and in the serous cavities of the brain; and indeed it may penetrate from its usual site of development in the mouth to any of the more distant organs.

The organism is commonly found as a diplococcus, though here and there short chains of four to six individuals may be detected. (Fig. 63.) The individual cells are more or less oval, or, more strictly speaking, lancet-shaped, for at one end they are commonly pointed. When joined in pairs the junction is always at the broad ends of the ovals, *never* at the pointed extremities. When in chains only the terminal cells are pointed, and then at their distal extremities.

As already stated, in preparations directly from the sputum or from the blood of animals a delicate capsule may frequently be seen surrounding them. Though fairly constant in preparations directly from the blood of animals and from the sputum or lungs of pneumonic patients, the capsule is but rarely observed in artificial cultures. Occasionally in cultures on blood-serum, in milk, and on agar-agar it can, according to some

authors, be detected; but this is by no means constant, or even frequent.

Even under the most favorable artificial conditions this organism grows but slowly and frequently not at all.

When successfully grown upon the different media it presents somewhat the following appearances:

On gelatin its development is very limited and often no growth at all occurs. This is probably due in part to the low temperature at which gelatin cultures must be kept. If development occurs, the growth appears as minute whitish or blue-white points on the plates. These very small colonies are round, finely granular, sharply circumscribed, and slightly elevated above the surface of the gelatin. They do not cause liquefaction of the gelatin.

If grown in slant- or stab-cultures, the surface-development is very limited; along the needle-track tiny whitish or bluish-white granules appear.

On nutrient agar-agar the colonies are almost transparent, more or less glistening, and very delicate in structure. On blood-serum development is more marked, though still extremely feeble, appearing as a cluster of isolated fine points growing closely side by side.

Growth on potato is not usually observed.

When grown in milk it commonly causes an acid reaction with coincident coagulation of the casein. Some varieties, especially non-virulent ones, do not coagulate milk.[1]

It is not motile.

It grows best at a temperature of from 35° to 38° C.

[1] Welch: loc. cit.

Below 24° C. there is usually no development, but in a few cases it has been seen to grow at as low a temperature as 18° C. Above 42° C. development is checked.

It grows as well without as with oxygen. It is therefore one of the facultative anaërobic forms.

Cultivation of this organism is most successful when the agar-agar-gelatin mixture of Guarniari is employed. (See this medium.)

It may be stained with the ordinary aniline staining-reagents. For demonstrating the capsule the method of Gram and the acetic-acid method give the best results. (See Stainings.)

This organism is conspicuous for the irregularity of its behavior when grown under artificial conditions: usually it loses its pathogenic properties after a few generations; but again this peculiarity may be retained for a much longer time. Not rarely it fails to grow after three or four transplantations on artificial media, though at times it may be carried through many generations.

Inoculation into Animals.—The results of inoculations with pure cultures of this organism are also conspicuous for their irregularity. When the organism is of full virulence the form of septicæmia just described is usually produced, but at times it is found to be totally devoid of pathogenic powers: between these extremes cultures may be obtained possessing every variation in the intensity of their disease-producing properties. The principal pathological conditions that may be produced by the inoculation of susceptible animals with this organism are, according to the degree of its virulence, acute septicæmia, spreading inflammatory exudations, and circumscribed abscesses. All three of these

conditions may sometimes be produced by inoculating rabbits with the same cultures in varying amounts.

Rabbits, mice, guinea-pigs, dogs, rats, cats, and sheep are susceptible to infection by this organism. Chickens and pigeons are insusceptible. Young animals, as a rule, are more easily infected than old ones. Rabbits and mice are the most susceptible of the animals used for experimental purposes, and in testing the virulence of a culture it is well to inoculate one of each, for the same culture may sometimes be virulent for mice and not for rabbits, and *vice versa*.

If the culture is virulent, intravascular or intraperitoneal injections into rabbits may produce rapid and fatal septicæmia; while subcutaneous inoculation of the same material may result in only a localized inflammatory process. On the other hand, subcutaneous inoculation of less virulent cultures may produce a local process, while intravenous inoculation may be without result. This organism is the cause of a number of pathological conditions in human beings that have not hitherto been considered as related to one another etiologically. It is always present in the inflamed area of the lung in acute fibrinous or lobar pneumonia; it is known to cause acute cerebro-spinal meningitis, endo- and peri-carditis, certain forms of pleuritis, arthritis and peri-arthritis, and otitis media.

b. SEPTICÆMIA CAUSED BY MICROCOCCUS TETRAGENUS.

Should the death of the animal not occur within the first twenty-eight to thirty hours after inoculation, but be postponed until between the fourth and eighth day, it may result from the invasion of the tissues by the organism now to be described, viz., *micrococcus tetragenus*.

This organism was discovered by Gaffky, and was subsequently described by Koch in the account of his experiments upon tuberculosis. It is often present in the saliva of healthy individuals and is commonly present in the sputum of tuberculous patients. Koch found it very frequently in the pulmonary cavities of phthisical patients. It, however, plays no part in the etiology of tuberculosis.

It is a small round coccus of about 1 μ transverse diameter. It is seen as single cells, joined in pairs, and in threes; but its most conspicuous grouping is in fours, from which arrangement it takes its name. In preparations made from cultures of this organism it is not rare to find, here and there, single bodies which are much larger than the other individuals in the field. Close inspection reveals them to be cells in the initial stage of division into twos and fours. A peculiarity of this organism is that the cells are bound together by a transparent gelatinous mass.

When cultivated artificially it grows very slowly.

Upon gelatin plates the colonies appear as round, sharply circumscribed, punctiform masses which are slightly elevated above the surface of the surrounding medium. Under a low magnifying power they are seen to be slightly granular and to present a more or less glassy lustre.

The colonies increase but little in size after the third or fourth day. If cultivated as stab-cultures in gelatin, there appears upon the surface at the point of inoculation a circumscribed white point, slightly elevated above the surface and limited to the immediate neighborhood of the point of inoculation. Down the needle-track the growth is not continuous, but appears in isolated, round,

dense white clumps or beads, which do not develop beyond very small points.

It does not liquefy gelatin.

Upon plates of nutrient agar-agar the colonies appear as small, almost transparent, round points, which have about the same color and appearance as a drop of egg-albumin; they are very slightly opaque. They are moist and glistening. They rarely develop to an extent exceeding 1 to 2 mm. in diameter.

Upon agar-agar as stab- or slant-cultures the surface-growth has more or less of a mucoid appearance. It is moist, glistening, and irregularly outlined. The outline of the growth depends upon the moisture of the agar-agar. It is slightly elevated above the surface of the medium.

In contradistinction to the gelatin stab-cultures, the growth in agar-agar is continuous along the track of the needle.

The growth on potato is a thick, irregular, slimy-looking patch.

The transparent mucilaginous substance which is seen to surround these organisms renders them coherent, so that efforts to take up a portion of a colony from the agar-agar or potato cultures result usually in drawing out fine, silky threads, consisting of organisms imbedded in the mucoid material.

The organism grows best at from 35° to 38° C., but can be cultivated at the ordinary room-temperature—about 20° C.

The growth under all conditions is slow.

It grows both in the presence of and without oxygen.

It is not motile.

It stains readily with all the ordinary aniline dyes.

In tissues its presence is readily demonstrated by the staining-method of Gram.

The grouping into fours is particularly well seen in sections from the organs of animals dead of this form of septicæmia. In such sections the organisms will always be found within the capillaries.

INOCULATION INTO ANIMALS.—To the naked eye no alteration can be seen in the organs of animals that have died as a result of inoculation with *micrococcus tetragenus;* but microscopic examination of cover-slip preparations from the blood and viscera reveals the presence of the organisms throughout the body—especially is this true of preparations from the spleen. White mice and guinea-pigs are susceptible to the disease. Gray mice, dogs, and rabbits are not susceptible to this form of septicæmia. Subsequent inoculation of healthy animals with a drop of blood, a bit of tissue, or a portion of a pure culture of this organism from the body of an animal dead of this disease, results in a reproduction of the conditions found in the dead animal from which the tissues or cultures were obtained.

It sometimes happens that in guinea-pigs which have been inoculated with this organism local pus-formations result, instead of a general septicæmia. The organisms will then be found in the pus-cavity.

CHAPTER XVIII.

Tuberculosis—Microscopic appearance of miliary tubercles—Encapsulation of tuberculous foci—Diffuse caseation—Cavity-formation—Primary infection—Modes of infection—Location of the bacilli in the tissues—Staining-peculiarities—Organisms with which *bacillus tuberculosis* may be confounded—Points of differentiation—Bacillus of influenza.

c. LOCAL OR GENERAL TUBERCULOSIS.

SHOULD the animal succumb to neither of the septic processes just described, then its death from tuberculosis may be reasonably expected.

When this disease is in progress alterations in the lymphatic glands nearest the site of inoculation may be detected by the touch in from two to four weeks. They will then be found enlarged. Though not constant, tumefaction and subsequent ulceration at the point of inoculation may be observed. Progressive emaciation, loss of appetite, and difficulty in respiration point to the existence of the general tuberculous process. Death ensues in from four to eight weeks after inoculation. At autopsy either general or local tuberculosis may be found. The expressions of tuberculosis are so manifold and in different animals vary so widely the one from the other, that no fixed law as to what will appear at autopsy can *à priori* be laid down.

The guinea-pig, which is best suited for this experiment because its susceptibility to tuberculosis is greater and more constant than that of other animals usually

found in the laboratory, presents, in the main, changes that are characterized by coagulation-necrosis and caseation. This is particularly the case when the infection is general—*i. e.*, when the process is of the acute miliary type; then the tissues of the liver and spleen present the most favorable field for the study of this pathological-anatomical alteration.

In general, the tubercular lesions can be divided into those of strictly focal character—*i. e.*, the miliary and the conglomerate tubercles—and those which are more diffuse. The latter lesions, although fundamentally of the same nature as the miliary tubercles, are much greater in extent and not so sharply circumscribed. These latter lesions play a more conspicuous *rôle* in the pathology of the disease than do the miliary nodules, although it is to the presence of the miliary nodules that the disease owes its name.

At autopsy the pathological manifestations of the disease are not infrequently seen to be confined to the seat of inoculation and to the neighboring lymphatic glands. These tissues then present all the characteristics of the tuberculous process in the stage of cheesy degeneration. When the disease is more general the degree of its extension varies. Sometimes the small gray nodules—miliary tubercles—are only to be seen with the naked eye in the tissues of the liver and spleen. Again, they may invade the lung, and frequently they are distributed over the serous membranes of the intestines, the lungs, the heart, and the brain. These gray nodules, as seen by the naked eye, vary in size from that of a pin-point to that of a hempseed, and, as a rule, are, in this stage, the result of the fusion of two or more smaller miliary foci. Though the two terms "miliary" and "conglomerate"

are employed for the description of the microscopic appearance of these nodules, yet it is very rarely that any condition other than that due to the fusion of several of these minute foci can be detected by the naked eye.

The miliary tubercles are of a pale gray color, with a white centre, are slightly elevated above the surface of the tissue in which they are located, and, as stated, vary considerably in dimensions, usually appearing as points which range in size from that of a pin-point to that of a pin-head. They are not only located upon the surface of the organs, but are distributed through the depths of the tissues. To the touch they sometimes present nothing characteristic, but when closely packed together in large numbers they usually give a mealy or sandy sensation to the hand passed over them. Stained sections of miliary tubercles present a distinctly characteristic appearance, and the disease may be diagnosticated by these histological changes alone, though the crucial test in the diagnosis is the demonstration of tubercle bacilli in these nodules.

Microscopic Appearance of Miliary Tubercles.—A miliary tubercle under a low magnifying power of the microscope presents somewhat the following appearance: there is a central pale area, evidently composed of necrotic tissue because of its incapacity for taking up the nuclear stains commonly employed. Scattered through this necrotic area may be seen granular masses irregular in size and shape; they take up the stains employed and are evidently fragments of cell-nuclei in course of destruction. Through the necrotic area may here and there be seen irregular lines, bands, or ridges, the remains of tissues not yet completely destroyed by the

necrotic process. Around the periphery of this area may sometimes be noticed large multinucleated cells, the nuclei of which are arranged about the periphery of the cell or grouped irregularly at its poles. The arrangement of these nuclei as observed in sections is usually oval, or somewhat crescentic. In tubercles from the human subject these large "giant-cells," as they are called, are quite common. They are much less frequent in tubercular tissues from lower animals.

Round about the central focus of necrosis is seen a more or less broad zone of closely packed small round and oval bodies, which stain readily but not homogeneously. They vary in size and shape, and are seen to be imbedded in a delicate network of fibrinous-looking tissue. This fibrin-like network in which these bodies lie, and which is a common accompaniment of giant-cell formation, is in part composed of fibrin, but is in the main, most probably, the remains of the interstitial fibrous tissue of the part. This zone of which we are speaking is the zone of so-called "granulation-tissue," and consists of leucocytes, granulation-cells, fibrin, and the fibrous remains of the organ; the irregularly oval, granular bodies which take up the stain are the nuclei of these cells. The zone of granulation-tissue surrounds the whole of the tuberculous process, and at its periphery fades gradually into the healthy surrounding tissues or fuses with a similar zone surrounding another tubercular focus. This may be taken as a description of the typical miliary tubercle.

Diffuse Caseation.—The diffuse caseation, as said, plays a more important *rôle* in the tuberculous lesion, both in the human and experimental forms, than does

the formation of miliary tubercles. In this a large area of tissue undergoes the same process of necrosis and caseation as the centre of the miliary tubercle. In certain tissues, notably the lungs and lymphatics, it is more marked than in others. In rabbits, particularly, all the changes in the lung frequently come under this head. When this is the case solid masses are found, sometimes as large as a pea, or involving even an entire lobe or the whole lung in some cases. They are of a whitish-yellow, opaque color, and on section are peculiarly dry and hard. Entire lymphatic glands may be changed in this way. The conditions which appear to be most favorable to the occurrence of this widespread caseation of the tissues are the simultaneous deposition of a large number of tubercle bacilli in them, and the involvement of a wide area instead of a single isolated point, as in the miliary tubercle. Necrosis is so rapid that time does not suffice for those reactive changes to take place in the tissues which result in the formation of the outer zone of the miliary tubercle. In other instances the entire caseous area is surrounded by a granulation-zone similar to that around the caseous centre of the miliary tubercles. It is of special importance to recognize the etiological connection between this diffuse caseation and the tubercle bacillus, because until its nature was accurately determined caseous pneumonia of the lungs formed the chief obstacle which many encountered in recognizing the specific infectiousness of tuberculosis.

CAVITY-FORMATION.—The production of cavities, a prominent feature in human tuberculosis, particularly of the lungs, is due to softening of the necrotic, caseous masses or of aggregations of miliary tuber-

cles. The material softens and is expelled, and a cavity remains. In the wall of this cavity the tuberculous changes still proceed, both as diffuse caseation and formation of miliary tubercles. The whole cavity with the reactive changes in the tissues of its walls may be properly conceived as a single gigantic tubercle, its wall forming a tissue very analogous to the outer zone of the single tubercle, the cavity itself corresponding to the caseous centre.

In animals used for experiment cavity-formation of this sort is very rare, owing to the greater resistance of the caseous tissue. That it is, however, possible to produce in rabbits pulmonary cavities in all physical respects similar to those seen in the human being has been most beautifully demonstrated by Prudden. He showed that when he had injected fluid cultures of *streptococcus pyogenes* into the trachea of rabbits already affected with tubercular consolidation of the lungs, the result of the mixed infection thus brought about was cavity-formation in eight out of nine lungs subjected to the conditions of the experiment; while in only one out of eleven did cavities form under the influence of the tubercle bacillus alone.[1]

In the contents and in the walls of tubercular cavities in man bacteria other than *bacillus tuberculosis* are found. It is to the influence of some of these, as we have seen, that diseases other than tuberculosis may sometimes be produced by the inoculation of animals with the sputum from such cases.

ENCAPSULATION OF TUBERCULAR FOCI.—It not

[1] Prudden: "Experimental Phthisis in Rabbits, with the Formation of Cavities," etc., Transactions of the Association of American Physicians, 1894, vol. ix. p. 166.

uncommonly occurs that round about a necrotic tuberculous focus there is formed a fibrous capsule which may completely shut off the diseased from the healthy tissue surrounding it; or a tuberculous focus may, through the resistance of the tissue in which it is located, be more or less completely isolated. In this condition the diseased foci may lie dormant for a long time and give no evidence of their existence, until they are caused to break through their envelopes by some disturbing cause. With the passage of the bacilli or their spores from such a focus into the vascular or lymphatic circulation the disease may become general.

It is to some such accident as this that the sudden appearance of general tubercular infection in subjects supposed to have recovered from the primary local manifestations may often be attributed. The breaking-down of old caseous lymphatic glands is a common example of this recurrence of tuberculosis.

PRIMARY INFECTION. — Primary infection occurs through either the vascular or lymphatic circulation. Through these channels the bacilli gain access to the tissues and become lodged in the finer capillary ramifications or in the more minute lymph-spaces. Here they find conditions favorable to their development, and in the course of their life-processes produce substances of a chemical nature which serve to bring about the death of the tissues in their immediate neighborhood. This tissue-death is probably the very first effect of the bacilli in the body, and represents the necrotic centre which can always be seen in the most minute tubercles. With the production progressive necrosis—for progressive it is, as it as long as the bacilli live and continue to

produce their poisonous products—there is in addition a reactive change in the surrounding tissues, which consists in the formation of a granulation-zone at the outer margins of the dying and dead tissue. This zone consists of small, round granulation-cells and of leucocytes, all of which are seen in the meshes of the finer fibrous tissues of the part. At the same time alterations are produced in the walls of the vessels of the locality; these tend to occlude them, and thus the process of tissue-death is favored by a diminution of the amount of nutrition brought to them. These changes may continue until eventually conglomerate tubercles, widespread caseation, or cavity-formation results; or from one cause or another the life-processes of the bacilli may be checked and recovery occur.

Modes of Infection.—Experimentally, tuberculosis may be produced in susceptible animals by subcutaneous inoculation, by direct injection into the circulation, by injection into the peritoneal cavity, by feeding of tuberculous material, by the introduction of the bacilli into the air-passages, and by inoculation into the anterior chamber of the eye.

In the human subject the most common portals of infection are, doubtless, the air-passages, the alimentary tract, and cutaneous wounds. When introduced subcutaneously the resulting process finds its most pronounced expression in the lymphatic system. The growing bacilli make their way into the lymphatic spaces of the loose cellular tissue, are taken up in the lymph-stream and deposited in the neighboring lymphatic glands. Here they may remain and give rise to no alteration other than that seen in the glands themselves; or they may pass on to neighboring glands,

and eventually be disseminated throughout the lymphatic system, ultimately reaching the vascular system.

Having gained access to the bloodvessels the results are the same as those following intravascular injection of the bacilli, namely: general tuberculosis quickly follows, with the production of miliary tubercles most conspicuous in the lungs and kidneys; less numerous in the spleen, liver, and bone-marrow.

When inhaled into the lungs, if conditions are favorable, multiplication of the bacilli quickly follows. Coincident with their growth they are mechanically pressed into the tissues of the lungs. As multiplication continues some are transported from the primary site of infection to healthy portions of the lung-tissue, where, through their development, the process is repeated.

In the same way infection by way of the alimentary tract is in the main due to the bacilli being forced by mechanical pressure into the walls of the intestines. Investigation has shown that lesions of the intestinal coats are not necessary for the entrance of tubercle bacilli from the lumen of the intestines into the internal organs and tissues. They may be transported from the intestinal tract into the lymphatics in the same way that the fat-droplets of the chyle find entrance into the lymphatic circulation.

Unlike most pathogenic organisms, the tubercle bacillus is believed to have the property of forming spores within the tissues. These spores, which are presumably highly resistant and not destroyed by drying, are thrown off from the lungs in the sputum of tuberculous patients in large numbers; and unless special precautions be taken to prevent it, the sputum becomes dried, is ground into dust, and sets free in the atmos-

phere the spores of tubercle bacilli which came with it from the lungs, and which have the property of exciting the disease in a certain number of persons who inhale them. The frequency of *pulmonary* tuberculosis points to this as one of the commonest sources and modes of infection in human beings. This opinion is borne out by statistical studies upon the disease, as well as by such evidence as Cornet[1] has produced upon the infective nature of dust taken from apartments occupied by persons suffering from pulmonary tuberculosis.

Location of the Bacilli in the Tissues.—The bacilli will be found most numerous in those tissues which are the seats of the active stage of the process.

In the initial stage of the disease the bacilli will be fewer in number than later. At this time only single rods may here and there be found; later they are more numerous; and, finally, when the process has advanced to a stage easily recognizable by the naked eye, they are found in the granulation-zones in clumps and scattered about in large numbers.

In the central necrotic masses, which consist of cell-detritus, it is rare that the organisms can be demonstrated microscopically. It is at the periphery of these areas and in the progressing granular zone that they are most frequently to be seen.

This apparent absence of the bacilli from the central necrotic area and often from old caseous tissues must not be taken, however, as evidence that these materials are not infective. As bacilli, they are difficult to demonstrate here because the probabilities are that at this stage of the process, owing to conditions unfavorable to their further growth, they are in the spore-

[1] Cornet: Zeitschrift für Hygiene, 1889, Bd. v. S. 191.

stage, a stage in which it is as yet impossible, with our present methods of staining, to render them visible. The facts that this tissue is infective, and that with it the disease can be reproduced in susceptible animals, speak for the accuracy of this assumption. A conspicuous example of this condition is seen in old scrofulous glands. These glands usually present a slow process, are commonly caseous, and always possess the property of producing the disease when introduced into the tissues of susceptible animals, and yet they are the most difficult of all tissues in which to demonstrate microscopically the presence of tubercle bacilli.

In tubercles containing giant-cells the bacilli can usually be demonstrated in the granular contents of these cells. Frequently they will be found accumulated at the pole of the cell opposite to that occupied by the nuclei, as if there existed an antagonism between the nuclei and the bacilli. In some of these cells, however, the distribution of the bacilli is seen to be irregular, and they will be found scattered among the nuclei as well as in the necrotic centre of the cell. As the number of bacilli in the giant-cell increases the cell itself is ultimately destroyed.

Tubercular tissues always contain the bacilli or their spores,[1] and are always capable of reproducing the disease when introduced into the body of a susceptible animal. From the tissues of this animal the bacilli may be obtained and cultivated artificially, and these cultures are capable of again producing the disease when further inoculated. Thus the postulates formulated by Koch, which are necessary to prove the

[1] The property of spore-formation is assumed, not proved.

etiological *rôle* of an organism in the production of a malady, are fulfilled.

THE TUBERCLE BACILLUS.

Of the three pathogenic organisms liable to occur in the sputum of a tuberculous subject, the tubercle bacillus gives us the greatest difficulty in our efforts at cultivation.

It is, in the strict sense of the word, a parasite, and finds conditions entirely favorable to its development only in the animal body. On ordinary artificial media the bacilli taken directly from the animal body grow only very imperfectly, or, in many cases, not at all. From this it seems probable that there is a difference in the nature of individual tubercle bacilli—some appearing to be capable of growth in the animal tissues only, while others are apparently possessed of the power to lead a limited saprophytic existence. It may be, therefore, that those bacilli which we obtain as artificial cultures from the animal body are offsprings of the more saprophytic varieties. At best, one never sees with the tubercle bacillus a saprophytic condition in any way comparable to that possessed by many of the other organisms with which we have to deal.

For the cultivation of *bacillus tuberculosis* directly from the tissues of the animal the method by which one obtains the best results is that recommended by Koch, viz., cultivation upon blood-serum. This organism is so strictly parasitic that even very slight variations in the conditions under which one endeavors to isolate it from the tissues may cause total failure. It is, therefore, necessary that the injunctions for obtaining it in pure culture should be carefully observed.

Preparation of Cultures from Tissues.—Under strictest antiseptic precautions remove from the animal the diseased organ—the liver, spleen, or a lymphatic gland being preferable. Place the tissue in a sterilized Petri dish, and dissect out with sterilized scissors and forceps the small tubercular nodules. Place each nodule upon the surface of the blood-serum, one nodule in each tube, and with a heavy, sterilized, looped platinum needle or spatula rub it carefully over the surface. It is best to dissect away twenty to thirty such tubercles and treat each in the same way. Some of the tubes will remain sterile, others may be contaminated by extraneous saprophytic organisms during the manipulation, while a few may give the result desired, viz., a growth of the tubercle bacillus itself.

The blood-serum upon which the organism is to be cultivated should be comparatively freshly prepared—that is, should not be dry.

After inoculating the tubes they should be carefully sealed to prevent evaporation and consequent drying. This is done by burning off the overhanging cotton plug in a gas-flame, and then impregnating the upper layers of the cotton with either sealing-wax or paraffin of a high melting-point; or by inserting over the burned end of the cotton plug a soft, closely fitting cork that has been sterilized in the steam sterilizer just before using (Ghriskey). This precaution is necessary because of the slow growth of the organism. Under the most favorable conditions tubercle bacilli directly from the animal body show no evidence of growth for about twelve days after inoculation upon blood-serum, and, as they must be retained during this time at the body-temperature—37.5° C.—

evaporation would take place very rapidly and the medium would become too dry for their development.

If these primary efforts result in the appearance of a culture of the bacilli, further cultivations may be made by taking up a bit of the colony, preferably a moderately large quantity, and transferring it to fresh serum, and this in turn is sealed up and retained at the same temperature. Once having obtained the organism in pure culture, its subsequent cultivation may be conducted upon the glycerin-agar-agar mixture—ordinary neutral nutrient agar-agar to which from 4 to 6 per cent. of glycerin has been added. This is a very favorable medium for the growth of this organism after it has accommodated itself to its saprophytic mode of existence, though blood-serum is perhaps the best medium to be employed in obtaining the first generation of the organism from tuberculous tissues.

The organism may be cultivated also on neutral milk to which 1 per cent. of agar-agar has been added, also upon the surface of potato, and likewise in meat-infusion bouillon containing from 4 to 6 per cent. of glycerin.

Cultures of the tubercle bacillus are characteristic in appearance—once having seen them there is little probability of subsequent mistake. They appear as dry masses, which may develop upon the surface of the medium either as flat scales or as coarse granular masses. They are never moist, and frequently have the appearance of dry meal spread upon the surface of the medium. In the lower part of the tube in which they are growing—*i. e.*, that part occupied by a few drops of fluid which has in part been squeezed from the medium during the process of solidification, and is in part water

of condensation—the colonies may be seen to float as a thin pellicle upon the surface of the fluid.

The individuals composing the growth adhere so tenaciously together that it is with the greatest difficulty they can be separated. In even the oldest and dryest cultures pulverization is impossible. The masses can only be separated and broken up by grinding in a mortar with the addition of some foreign substance, such as very fine, sterilized sand, dust, etc.

The cultures are of a dirty-drab or brownish-gray color when seen on serum or glycerin-agar-agar.

On potato they grow in practically the same way, though the development is much more limited. On this medium they are of nearly the same color as the potato on which they are growing. When cultivated for a time on potato they are said to lose their pathogenic properties.

On milk-agar-agar they are of so nearly the same color as the medium that, unless they are growing as characteristic mealy-looking masses, considerably elevated above the surface, their presence is less conspicuous than when on other media.

In bouillon they grow as a thin pellicle on the surface. This may fall to the bottom of the fluid and continue to develop, its place on the surface being taken by a second pellicle.

The tubercle bacillus does not develop on gelatin because of the low temperature at which this medium must be used.

Microscopic Appearance of Bacillus Tuberculosis.—Microscopically the organism itself is a delicate rod, usually somewhat beaded in its structure, though rarely it is seen to be homogeneous. It is either

quite straight, or somewhat curved or bent on its long axis. In some preparations involution-forms, consisting of rods a little clubbed at one extremity or slightly bulging at different points, may be detected. Branching forms of this organism have been described. It varies in length—sometimes being seen in very short segments, again much longer, though never as long threads. Usually its length varies from 2 to 5 μ. It is commonly described as being in length about one-fourth to one-half the diameter of a red blood-corpuscle. It is very slender. (See Fig. 62.)

These rods usually present, as has been said, an appearance of alternate stained and colorless portions. The latter portions are believed to be the spores of the organism, though as yet no incontestable proof of this opinion has been presented. At times these colorless portions are seen to bulge slightly beyond the contour of the rod, and in this way give to the rods the beaded appearance so commonly ascribed to them.

STAINING-PECULIARITIES.—A peculiarity of this organism is its behavior toward staining-reagents, and by this means alone it may be easily recognized. The tubercle bacillus does not stain by the ordinary methods. It possesses some peculiarity in its composition that renders it proof against the simpler staining processes. It is therefore necessary that more energetic and penetrating reagents than the ordinary watery solutions should be employed. Experience has taught us that certain substances not only increase the solubility of the aniline dyes, but by their presence the penetration of the coloring-agents is very much increased. Two of these are aniline oil and carbolic acid. They are employed in the solutions to about the point of satura-

tion. (For the exact proportions, see chapter on Staining-reagents.)

Under the influence of heat these solutions are seen to stain all bacteria very intensely—the tubercle bacilli as well as other forms. If we subject our preparation, which may contain a mixture of tubercle bacilli and other species, to the action of decolorizing-agents, another peculiarity of the tubercle bacillus will be observed. While all other organisms in the preparation give up their color and become invisible, the tubercle bacillus retains it with marked tenacity. It stains with great difficulty; but once stained it retains the color even under the action of strong decolorizing-agents.

ORGANISMS WITH WHICH BACILLUS TUBERCULOSIS MAY BE CONFUSED.

DIFFERENTIAL DIAGNOSIS.—While its peculiar micro-chemical reaction is usually considered to be diagnostic of *bacillus tuberculosis*, it is well to remember that there are at least three other species of bacilli which, when similarly treated, react in the same way. It is of importance to bear this point in mind, particularly in the microscopic examination of urine and pathological secretions from the genito-urinary tract and from the rectum, for of the three species two are frequently found in these localities, viz., the so-called *smegma bacillus*, located in the smegma and often seen beneath the prepuce and upon the vulva, both normally and in disease, and the so-called *bacillus of syphilis*, described by Lustgarten as contained in syphilitic manifestations, particularly in primary sores. The third organism of this group—the

bacillus of leprosy—because of its rarity is not so likely to cause error in the diagnosis of pathological conditions occurring in these localities.

According to Hueppe, the differential diagnosis between the four organisms depends upon the following reactions: when stained by the carbol-fuchsin method commonly employed in staining the tubercle bacillus the syphilis bacillus becomes almost instantly decolorized by treatment with mineral acids, particularly sulphuric acid, whereas the smegma bacillus resists such treatment for a much longer time, and the lepra and tubercle bacillus for a still longer time. On the other hand, if decolorization is practised with alcohol, instead of acids, the smegma bacillus is the first to lose its color. The bacillus tuberculosis and the bacillus of leprosy are conspicuously retentive of their color even after treatment with both acids and alcohol. To differentiate, then, between the four organisms he recommends the following order of procedure, based on the above reactions:

1. Treat the preparation, stained with carbol-fuchsin, with dilute sulphuric acid; the syphilis bacillus becomes decolorized, the reaction being almost instantaneous.

2. If it is not at once decolorized, treat with alcohol; if it is the smegma bacillus, this will rob it of its color.

3. If it is still not decolorized, it is either the lepra or tubercle bacillus.

Grethe recommends[1] the following as a trustworthy means of distinguishing between the tubercle bacillus and the smegma bacillus: stain in hot carbol-fuchsin solution, wash in water, and treat the preparation with a saturated solution of methylene-blue in *alcohol*. If the question-

[1] Fortschritte der Medicin., 1896, No. 9.

able organism is the tubercle bacillus, it retains its *red* color; if the smegma bacillus, the red color is dissolved by the alcohol and the blue color is substituted for it.

The differential diagnosis between the tubercle bacillus and the lepra bacillus is less satisfactory; they both take the same stains, and both retain them or give them up under treatment with the same decolorizers. The results of investigations, however, indicate differences in the *rate* of staining and decolorization, and it is stated by many of those who have compared the two organisms that the lepra bacillus takes up stain very much more readily than does the tubercle bacillus, often staining perfectly after an exposure of only a few minutes to cold watery solutions of the dyes; but when once stained it retains its color much more tenaciously when acted upon by decolorizing-agents than does the latter organism.

According to Baumgarten, the lepra bacillus is stained by an exposure of six to seven minutes to a cold, saturated watery solution of fuchsin, and retains the stain when subsequently treated with acid alcohol (nitric acid, 1 part; alcohol, 10 parts). By similar treatment for the same length of time *bacillus tuberculosis* does not ordinarily become stained.

These points, particularly what has been said with reference to the smegma bacillus and the bacillus of syphilis, are of much practical importance, and should always be borne in mind in connection with the microscopic examination of materials to which these organisms are likely to gain access. It is hardly necessary to say that in the examination of sputum and pathological fluids from other parts of the body the tubercle bacillus is, of the four organisms, always the one most commonly

encountered, while the organism described by Lustgarten as the bacillus of syphilis is seen so rarely that many trustworthy investigators question its existence as a species distinct from the ordinary smegma bacillus.

TUBERCULIN.—The filtered products of growth from old fluid cultures of the tubercle bacillus represent what is known as *tuberculin*—a group of proteid substances possessing most interesting properties. When injected subcutaneously into healthy subjects tuberculin has no effect; but when introduced into the body of a tuberculous person or animal a pronounced systemic reaction results, consisting of sudden but temporary elevation of temperature, with, at the same time, the occurrence of marked hyperæmia about the tuberculous focus, a change histologically analogous to that seen in the primary stages of acute inflammation. This zone of hyperæmia, with the coincident exudation and infiltration of cellular elements, probably aids in the isolation or casting off of the tuberculous nodule, the inflammatory zone forming, so to speak, a line of demarcation between the diseased and healthy tissue.

As a curative agent for the treatment of tuberculosis, tuberculin has not merited the confidence that was at first accorded to it. Its field of usefulness is now almost limited to the diagnosis of obscure cases, and even for this purpose it is less frequently employed than formerly.

In veterinary medicine it has proved much more trustworthy as a diagnostic aid, and is practically everywhere in use for the detection of incipient tuberculosis, especially in cattle.

SUSCEPTIBILITY OF ANIMALS TO TUBERCULOSIS.—

The animals that are known to be susceptible to tuberculosis are man, apes, cattle, horses, sheep, guinea-pigs, pigeons, rabbits, cats, and field-mice. White mice, dogs, and rats possess immunity from the disease.

We have reviewed the three common pathogenic organisms with which we may come in contact in the sputum of tuberculous individuals. Occasionally other forms may be present. The pyogenic forms are not rarely found, and for some time after an attack of diphtheria the bacillus of Löffler is demonstrable in the pharynx, so that it, too, may be present under exceptional circumstances.

From time to time fowls are known to suffer from a form of tuberculosis that in a number of ways suggests human or mammalian tuberculosis. The bacillus causing the disease, the so-called bacillus of fowl tuberculosis, *bacillus tuberculosis avium*, while simulating the genuine *bacillus tuberculosis* morphologically, differs from it both in cultural and pathogenic peculiarities. Thus, for instance, it develops into much longer and somewhat thinner threads; grows rapidly on media without glycerin or glucose; does not grow on potato; develops as well at from 42° to 43° C. as at 37° to 38° C.;[1] its virulence is not diminished by cultivation at 43° C.; development on artificial media begins in from six to eight days after inoculation; young cultures on solid media are whitish, soft, and moist, becoming yellowish and slimy with age; it is somewhat more resistant to drying and high temperatures than the bacillus of mammalian tuberculosis; the results of its patho-

[1] The normal body-temperature of fowls ranges between 41.5° and 42.5° C.

genic activities are almost always chronic, are rarely located in the lungs or intestines, but are especially frequent in the liver and spleen; the lesions are conspicuously rich in bacilli, do not show the central necrotic area that characterizes the mammalian tubercle; the disease is transmissible from the hen to the embryo chick; the only susceptible mammal is the rabbit; the guinea-pig and dog are naturally immune; it has the same micro-chemical staining-reactions as mammalian bacillus tuberculosis; it has never been certainly detected in human tuberculosis.

Some are inclined to regard this organism as but a variety of the genuine *bacillus tuberculosis*, and it is not unreasonable to believe that the sojourn of that organism in the body of a refractory animal, whose normal temperature is so high as that of the fowl, when not fatal to the organism, might result in striking modifications of certain of its biological functions. In fact, Nocard[1] has shown that if the genuine *bacillus tuberculosis* from man be left in the peritoneal cavity of chickens (by the collodion-sac method of Metschnikoff, Roux, and Sallembini, which see) for from five to eighth months, they will, by the end of this time, have become so modified in their biological peculiarities as to simulate very closely the bacillus of fowl tuberculosis.

Theobald Smith[2] has called attention to certain very conspicuous differences that may be observed between the bacilli obtained from human and those from bovine tuberculosis.

Anatomical lesions very suggestive of, though not

[1] Nocard: Annales de l'Institut Pasteur, 1898, p. 561.

[2] Smith: Transactions Association of American Physicians, 1896, vol. xi. p. 75.

identical with, those produced by *bacillus tuberculosis* have also from time to time been observed in mice, rats, guinea-pigs, rabbits, cats, goats, bovines, hogs, and man. They do not appear to be of a specific nature as regards etiology, for the reason that different authors have described different species of bacilli as the causative agents. These conditions suggest tuberculosis only by the more superficial character of the lesions, for in no instance have the organisms detected been in any way similar to the genuine *bacillus tuberculosis*. These affections usually pass under the name pseudo-tuberculosis.

THE BACILLUS OF INFLUENZA.

An important historic epidemic disease, on the nature of which much light has been shed through modern methods of investigation, is influenza. Quoting Hirsch, the first trustworthy literary records that we have of this disease date from the early part of the twelfth century.

Between 1173 and 1874 it made its epidemic or pandemic appearance on eighty-six different occasions. Its first appearance in this country was in Massachusetts in 1627; since that time there have been twenty-two visitations of influenza to the United States. The pandemic of 1889–'90, the most severe for a long time, appears to have originated in Central Asia and to have spread pretty much over the entire civilized world. The advent of influenza in a community is always remarkable for its astonishing rate of transmission from person to person and its dissemination over wide areas.

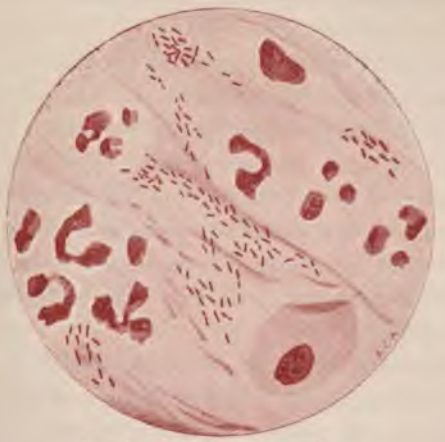
During the recent pandemic investigations having for their object the discovery of its cause were instituted, with the result of demonstrating in the catarrhal

secretions from the air-passages a micro-organism that is claimed to stand in causal relation to influenza.

This organism, *bacillus influenzæ*, as it is called, was discovered, isolated, cultivated, and described by R. Pfeiffer.

It is a very small, slender, non-spore-forming, non-motile, aërobic bacillus, occurring singly and in pairs, joined end to end. It stains with watery solutions of the ordinary basic aniline dyes; somewhat better with alkaline methylene-blue, but best when treated for five minutes with a dilution of Ziehl's carbol-fuchsin in water (the color of the solution should be pale red). (Fig. 64.) It is decolorized by the method of Gram.

FIG. 64.



Bacillus of influenza in sputum.

It develops only at temperatures ranging from 26° to 43° C. Its optimum temperature for growth is 37° C. It possesses the peculiarity of developing upon only those artificial culture-media to which blood or

blood-coloring-matter has been added. Its cultivation is best conducted and its development most satisfactorily observed by the following procedure: over the surface of a slanted agar tube or over agar-agar solidified in a Petri dish smear a small quantity of sterile blood (not blood-serum). A bit of the mucus from the sputum of the influenza patient is then taken up with sterilized forceps or on a sterilized wire loop, rinsed in sterile bouillon or water and rubbed over the surface of the prepared agar-agar. The plate or tube is then placed in the incubator at 37° to 38° C. If influenza bacilli be present, they will develop as minute, transparent, watery colonies that are without structure, and which resemble somewhat minute drops of dew. They are discrete and show little or no tendency to coalesce.

If a small bit of mucus be rubbed over the surface of ordinary nutrient agar-agar, no such colonies develop. In making the diagnosis by this method cultures on both agar-agar containing blood (not blood-serum) and agar-agar containing no blood should always be made, for the reason that growth of these peculiar colonies in the former and no such growth in the latter are evidence that one is dealing with materials from a case of influenza.

The organism may also be cultivated in bouillon to which blood has been added, if kept at body-temperature. The growth appears as whitish flakes. Since this organism is a strict aërobe, its cultivation can only be conducted on the surface of the medium used—*i. e.*, where it has freest access to oxygen. It is therefore inadvisable to prepare plates in the usual way. When its cultivation is attempted in bouillon it is recom-

mended, in order to favor the free diffusion of oxygen, that the depth of fluid be very shallow.

Contrary to what might be supposed, the bacillus of influenza has very little tenacity of life outside of the diseased body. It is destroyed in from two to three hours by rapid drying, and in from eight to twenty-four hours when dried more slowly. Cultures retain their vitality for from two to three weeks. The organism dies in water in a little over a day. As a result of these observations, Pfeiffer does not believe the disease to be disseminated by either the air or the water, but rather by direct infection from the catarrhal secretions of the patients.

This organism has not been found outside of the human body. In the influenza patient it is present in the catarrhal secretions, bronchial mucous membrane, and the diseased lung-tissues. It may be demonstrated microscopically in the mucus by cover-slip preparations made in the usual way and stained with diluted carbol-fuchsin, referred to above. In the tissues it may be demonstrated in sections stained in the same solution. In the sputum the bacilli are found as masses and as scattered cells. (See Fig. 64.) They are also found within the bodies of leucocytes, especially in the later stages of the disease when convalescence has set in; at this time they appear as very small, irregular, evidently degenerated bacilli within white blood-corpuscles. They are also present in the nasal secretions.

At autopsies it is advisable to cut out pieces of the diseased tissue about the size of a pea or a bean, break them up in a small quantity of sterile water or bouillon, and make the cultures from this infusion.

By this procedure two advantages are gained: first, a dilution of the number of bacteria present; and, secondly, the tissue furnishes the amount of hæmoglobin necessary for the growth of the organism. Under these circumstances it is, of course, not necessary to make a further addition of blood to the culture-medium.

The only animal that has been found susceptible to inoculation with this organism is the monkey. By intratracheal injection Pfeiffer succeeded in causing a toxic condition that proved fatal. He does not regard the death of the animals as due to infection, but rather to intoxication. The disease, as seen in man, has not been reproduced in animals.

CHAPTER XIX.

Glanders—Characteristics of the disease—Histological structure of the glanders nodule—Susceptibility of different animals to glanders—The bacillus of glanders; its morphological and cultural peculiarities—Diagnosis of glanders.

SYNONYMS: Rotz (Ger.), Morve (Fr.).

The disease is generally known as glanders when the mucous membrane of the nostrils is affected, and as farcy when the subcutaneous lymphatics are the principal sites of involvement.

Though most commonly seen in the horse and ass, glanders is not rarely met with in other animals, and is occasionally encountered in man. When occurring spontaneously in the horse its primary seat is usually upon the mucous membrane of the nostrils. It appears in the form of small gray nodules, about which the membrane is congested and swollen. These nodules ultimately coalesce to form ulcers. There is a profuse slimy discharge from the nostrils during the course of the disease. The primary lesion may extend from its seat in the nose to the mouth, larynx, trachea, and ultimately to the lungs. Its secondary manifestations are observed along the lymphatics that communicate with the initial focus; in the lymphatic glands, and as metastatic foci in the internal organs. Less frequently the disease is seen to begin in the skin, particularly in the region of the neck and breast. When in this locality the subcutaneous lymphatics become involved, and are con-

verted into indurated, knotty cords—"farcy-buds"—easily discernible from without.

In man it usually occurs in individuals who have been in attendance upon animals affected with the disease. It may occur upon the mucous membrane of the nares; but its most frequent expressions are in the skin and muscles, where appear abscesses, phlegmons, erysipelas-like inflammations, and local necrosis closely resembling carbuncles. Metastases to the lungs, kidneys, and testicles, as in the horse, may also be seen.

When occurring upon the mucous membrane glanders is characterized by the presence of gray nodules, about as large as a pin-head, that closely resemble miliary tubercles in their naked-eye appearance. These consist histologically of granulation-tissue—*i. e.*, of small round cells, very similar to proliferating leucocytes—of some lymph-cells, and, in the earliest stages, of a small portion of necrotic tissue. As they grow older, and the process advances, there is a tendency to central necrosis, with the ultimate formation of a soft, yellow, creamy, pus-like material. Though strikingly like miliary tubercles in certain respects in the early stages, they present, nevertheless, decided points of difference when examined more in detail.

The round-cell infiltration of the glanders nodule consists essentially of polynuclear leucocytes, while that of the miliary tubercle partakes more of the nature of a lymphocytic infiltration; in the later stages of the process the glanders nodule breaks down into a soft creamy matter, very analogous to ordinary pus, while in the later stages of the miliary tubercle the tendency is to an amalgamation of its histological constituents,

and ultimately to necrosis with caseation. The giant-cell formation common to tuberculosis is never seen in the glanders nodule. As Baumgarten aptly puts it: "The pathological manifestations of glanders, from the histological aspect, stand midway between the *acute purulent* and the *chronic* inflammatory processes."[1] Evidently these differences are only to be explained by differences in the nature of the causes that underlie the several affections. We have studied the characteristics of *bacillus tuberculosis;* we shall now take up the bacillus of glanders and note the striking differences between them.

THE BACILLUS OF GLANDERS (BACILLUS MALLEI).

In 1882 Löffler and Schütz discovered in the diseased tissues of animals suffering from glanders a bacillus that, when isolated in pure culture and inoculated into susceptible animals, possesses the property of reproducing the disease with all its clinical and pathological manifestations. It is therefore the cause of the disease.

This organism is a rod, with rounded or slightly pointed ends. It usually stains somewhat irregularly. (See Fig. 65.) When examined in stained preparations its continuity is marked by alternating darkly and lightly stained areas. It is usually seen as a single rod, but may occur in pairs, and less frequently in longer filaments.

The question as to its spore-forming property is still an open one, though the weight of evidence is in oppo-

[1] For a further discussion of the pathology and pathogenesis of this disease, see Lehrbuch der pathologischen Mykologie, by Baumgarten, 1890. See, also, Wright: "The Histological Lesions of Acute Glanders in Man," Journal of Experimental Medicine, vol. i. p. 577.

sition to the opinion that it possesses this peculiarity. Certain observers claim to have demonstrated spores in the bacilli by particular methods of staining; but this statement can have but little weight when compared with the behavior of the organism when subjected to more conclusive tests. For example, it does not, at any stage of development, resist exposure to 3 per cent.

FIG. 65.

Bacillus of glanders (*bacillus mallei*), from culture.

carbolic acid solution for longer than five minutes, nor to 1 : 5000 sublimate solution for more than two minutes. It is destroyed in ten minutes in some experiments, and in five in others, by a temperature of 55° C.; and when dried it loses its vitality, according to different observers, in from thirty to forty days; all of which speak directly against this being a spore-bearing bacillus.

It is not motile, and does not therefore possess

It grows readily on ordinary nutrient media at from 25° to 38° C.

Upon nutrient agar-agar, both with and without glycerin, it appears as a moist, opaque, glazed layer, with nothing characteristic about it. This is true both for smear-cultures and for single colonies.

Its growth on gelatin is much less voluminous than on media that can be kept at higher temperature, though it does grow on this media at room-temperature without causing liquefaction.

Its growth on blood-serum is in the form of a moist, opaque, slimy layer, inclining to a yellowish or dirty, brownish-yellow tinge. It does not liquefy the serum.

On potato its growth is moderately rapid, appearing in from twenty-four to thirty-six hours at 37° C. as a moist, amber-yellow, transparent deposit having somewhat the appearance of honey; this becomes deeper in color and denser in consistence as growth progresses, and finally takes on a reddish-brown color; at the same time the potato about it becomes darkened.

In bouillon it causes diffuse clouding, with ultimately the formation of a more or less tenacious or ropy sediment.

In milk to which a little litmus has been added it causes the blue color to become red or reddish in from four to five days, and quite red after two weeks at 37° C. At the same time the milk separates into clear whey and a firm clot of casein.

Its reactions to heat are very interesting. At 42° C. it will often grow for twenty days or more. It will not grow at 43° C., and if exposed to this temperature for forty-eight hours it is destroyed. It is killed in five

hours when exposed to 50° C., and in five minutes by 55° C.

It grows both with and without oxygen; it is therefore *facultative* as regards its relation to this gas.

On cover-slips it stains readily with all the basic aniline dyes, and, as a rule, as stated, presents conspicuous irregularities in the way that it takes up the dyes, being usually marked by deeply stained areas that alternate with points at which it either does not stain at all or only slightly.

The animals susceptible to infection by this organism are horses, asses, field-mice, guinea-pigs, and cats. Baumgarten records cases of infection in lions and tigers that were fed, in menageries, with flesh from horses affected with the disease. Rabbits are but slightly susceptible; dogs and sheep still less so. Man is susceptible, and infection not rarely terminates fatally. White mice, common gray house-mice, rats, cattle, and hogs are insusceptible.

Inoculation Experiments.—The most favorable animal upon which to study the pathogenic properties of this organism in the laboratory is the common field-mouse. When inoculated subcutaneously with a small portion of a pure culture of the glanders bacillus death ensues in about seventy-two hours. The most conspicuous tissue-changes will be enlargement of the spleen, which is at the same time, almost constantly, studded with minute gray nodules, the typical glanders nodule. They are rarely present in the lungs, but may frequently be seen in the liver. From these nodules the glanders bacillus may be obtained in pure culture. With the exception of the characteristic nodules, the disease as seen in this animal presents none of the features that

it displays in the horse and ass. The clinical and pathological manifestations resulting from inoculation of guinea-pigs are much more faithful reproductions. The animal lives usually from six to eight weeks after inoculation, and during this time becomes affected with a group of most interesting and peculiar pathological processes. The specific inflammatory condition of the mucous membrane of the nostrils is almost always present. The joints become swollen and infiltrated to such an extent as often to interfere with the use of the legs. In male animals the testicles become enormously distended with pus, and on closer examination a true orchitis and epididymitis are seen to be present. The internal organs, particularly the lungs, kidneys, spleen, and liver, are usually the seat of the nodular formations characteristic of the disease. From all of these disease-foci the bacillus causing them can be isolated in pure culture.

Staining in Tissues.—Though always present in the diseased tissues, considerable trouble is usually experienced in demonstrating the bacteria by staining-methods. The difficulty is due to the fact that the bacilli are very easily decolorized, and in tissues stained by the ordinary processes are robbed of their color even by the alcohol with which the tissue is rinsed and dehydrated. If we will remember not to employ concentrated stains, and not to expose the sections to the stains for too long a time, but little treatment with decolorizing-agents is necessary, and very satisfactory preparations will be obtained. A number of methods have been suggested for staining the glanders bacilli in tissues, and if what has been said will be borne in mind, no difficulty should be experienced.

Two satisfactory methods that we have used for this

purpose, though perhaps no better than some of the others, are as follows:

a. Transfer the sections from alcohol to distilled water. This lessens the intensity with which the stain subsequently takes hold of the tissues, by diminishing the activity of the diffusion that would occur if they were placed from alcohol into watery solutions of the dyes. Transfer from distilled water to the slide, absorb all water with blotting-paper, and stain with two or three drops of

Carbol-fuchsin	10 c.c.
Distilled water	100 c.c.

for thirty minutes; absorb all superfluous stain with blotting-paper, and wash the section three times with 0.3 per cent. acetic acid, not allowing the acid to act for more than ten seconds each time. Remove all acid from the section by carefully washing in distilled water; absorb all water by gentle pressure with blotting-paper; and finally, at *very moderate heat*, or with a small bellows (Kühne), dry the section completely on the slide. When dried clear in xylol and mount in xylol balsam.

b. Transfer the sections from alcohol to distilled water; from water to the dilute fuchsin solution, and gently warm (about 50° C.) for fifteen to twenty minutes. Transfer sections from the staining-solution to the slide, absorb all superfluous stain with blotting-paper, and then treat them with 1 per cent. acetic acid from one-half to three-quarters of a minute. Remove all trace of acid with distilled water, absorb all water by gentle pressure with blotting-paper, and then treat the sections with absolute alcohol by allowing it to flow

over them drop by drop. For small sections three or four drops are sufficient. Under no circumstances should the alcohol be allowed to act for more than one-quarter of a minute. Clear in xylol and mount in xylol balsam.

By method *b* the tissues are better preserved than by method *a*, by which they are dried.

Very good preparations are also obtained by the use of Löffler's alkaline methylene-blue, if care be taken not to stain for too long a time nor to decolorize too energetically with alcohol.

No method of contrast-stain for this organism in tissues has been devised.

In properly stained tissues the bacilli will be found most numerous in the centre of the nodules, becoming fewer as we approach the periphery. They usually lie between the cells, but at times may be seen almost filling some of the epithelial cells, of which the nodule contains more or less. They are always present in these nodules in the tissues; they are rarely present in the blood, and, if so, in only small numbers.

DIAGNOSIS OF THE DISEASE BY THE METHOD OF STRAUSS.—From what has been said, the diagnosis of glanders by routine bacteriological methods is certain and relatively easy, but requires time. In clinical work it is of great importance for the diagnosis to be established as quickly as possible. With this in view Strauss devised a method that has given entirely satisfactory results. It consists in introducing into the peritoneal cavity of a *male* guinea-pig a bit of the suspected tissue or culture. If it be from a genuine case of glanders, the testicles begin to swell in about thirty hours, and as this proceeds the skin over them becomes red and

shining, desquamation occurs, evidences of pus-formation are seen, and, indeed, the abscess (purulent orchitis) often breaks through the skin. The diagnostic sign is the tumefaction of the testicles.

MALLEIN.—The filtered products of growth of the glanders bacillus in fluid media represent what is known as *mallein*—a group of compounds that bear to glanders pretty much the same relation that tuberculin bears to tuberculosis. It is used with considerable success as a diagnostic aid in detecting the existence or absence of deep-seated manifestations of the disease, the glanderous animal reacting to subcutaneous injections of mallein in from four to ten hours, while an animal not so affected gives no such reactions.

Mallein is prepared from old glycerin-bouillon cultures of the glanders bacillus by steaming them for several hours in the sterilizer, after which they are filtered through unglazed porcelain.

CHAPTER XX.

Bacillus diphtheriæ—Its isolation and cultivation—Morphological and cultural peculiarities—Pathogenic properties—Variations in virulence—Pseudo-diphtheria bacilli.

From the gray-white deposit on the fauces of a diphtheritic patient prepare a series of cultures in the following way:

Have at hand five or six tubes of Löffler's blood-serum mixture. (See chapter on Media.) Pass a stout platinum needle, which has been sterilized, into the membrane and twist it around once or twice, or brush it gently over the surface of the membrane. Without touching it against anything else rub it carefully over the surface of one of the serum-tubes; without sterilizing it pass it over the surface of the second, then the third, fourth, and fifth tubes. Place these tubes in the incubator. Then prepare cover-slips from scrapings from the membrane on the fauces. If the case is one of true diphtheria, the tubes will be ready for examination on the following day.

The reason that plates are not made in the regular way in this examination is that the bacillus of diphtheria develops much more luxuriantly on the serum mixture, from which plates cannot be made, than it does on the media from which they can be made. The method employed, however, insures a dilution in the number of organisms present, and this, in addition to the fact that the blood-serum mixture is a much more

favorable medium for the rapid development of the diphtheria bacillus than of the other organisms present, makes its isolation by this method a matter of but little difficulty.

After twenty-four hours in the incubator the tubes present a characteristic appearance. Their surfaces are marked by more or less irregular patches of a white or cream-colored growth, which is usually more dense at the centre than at the periphery. Except now and then, when a few orange-colored colonies may be seen, these large irregular patches are the most conspicuous objects on the surface of the serum. Occasionally, almost nothing else appears.

The cover-slips made from the membrane at the time the cultures were prepared will be found on microscopic examination to present, in many cases, a great variety of organisms; but conspicuous among them will be noticed slightly curved bacilli of irregular size and outline. In some cases they will be more or less clubbed at one or both ends; sometimes they appear spindle in shape, again as curved wedges; now and then they are irregularly segmented. They are rarely or never regular in outline. If the preparation has been stained with Löffler's alkaline methylene-blue solution, many of these irregular rods are seen to be marked by circumscribed points in their protoplasm which stain very intensely—they appear almost black. This irregularity in outline is the morphological characteristic of *bacillus diphtheriæ* of Löffler.

It must be remembered, however, that the diagnosis of diphtheria should not under all circumstances be made from the examination of cover-slip preparations alone, especially when they are stained only by the

usual method—*i. e.*, with Löffler's methylene-blue. There are other organisms present in the mouth-cavity, particularly in the mouths of persons having decayed teeth, the morphology of which is so like that of the bacillus of diphtheria that they might easily be mistaken for that organism if subjected to only the usual method of microscopic examination; and again, the genuine diphtheria bacillus is sometimes found in the mouth-cavities of healthy persons in attendance upon diphtheria cases, such persons being at the time insusceptible to the pathogenic activities of the organism. In the vast majority of instances, however, where the clinical condition of the patient justifies a suspicion of diphtheria, a microscopic examination alone of the deposit in the throat will serve to confirm or contradict this opinion, and such examinations very frequently reveal the diphtheritic nature, etiologically speaking, of mild conditions of the throat which are not associated with grave constitutional manifestations.

Bacillus diphtheriæ, discovered microscopically by Klebs, and isolated in pure culture and proved to stand in causal relation to diphtheria by Löffler, can readily be identified by its cultural peculiarities and by its pathogenic activity when introduced into tissues of susceptible animals. In guinea-pigs and kittens the results of its growth are histologically identical with those found in the bodies of human beings who have died of diphtheria.

When studied in pure culture its morphological and cultural peculiarities are as follows:

Morphology.—As obtained directly from the diphtheritic deposit in the throat of an individual sick of the disease, it is sometimes comparatively regular in

shape, appearing as straight or slightly curved rods with more or less pointed ends. More frequently, however, spindle- and club-shapes occur, and not rarely many of these rods stain irregularly: in some of them very deeply stained round or oval points can be detected.

When *cultures* are examined microscopically it is especially characteristic to find irregular, bizarre forms, such as rods with one or both ends swollen, and very frequently rods broken at irregular intervals into short, sharply defined segments, either round, oval, or with straight sides. Some forms stain uniformly, others in various irregular ways, the most common being the appearance of deeply stained granules in a lightly stained bacillus.

By a series of studies upon this organism when cultivated under artificial conditions we have found that its form and size depend very largely upon the nature of its environment. That is to say, its morphology is always more regular, and it is smaller on glycerin-agar-agar than on other media used for its cultivation; while upon Löffler's blood-serum the other extremes of development appear: here one sees, instead of the very short, spindle-, lancet-, club-shaped, always segmented and regularly staining forms as seen upon glycerin-agar-agar, long, sometimes extremely slender, sometimes thicker, irregularly staining threads that are usually clubbed and frequently pointed at their extremities. They are, as a rule, marked by areas that stain more intensely than does the rest of the rod, and at times they may be a little swollen at the centre. These differences are so conspicuous that microscopic preparations from cultures from the same source, but cultivated in the one case on glycerin-agar-agar and in the other upon blood-

serum, when placed side by side would hardly be recognized as of the same organism, unless its peculiar behavior under these circumstances was already known. Another peculiar variation is that observed upon very slightly acid blood-serum. Here the rods appear swollen, and are usually contracted to oval or short, oblong bodies, which stain very faintly, and in which are usually located one or two very deeply staining round or oval points. Various authors have called attention to branching forms of this organism that are occasionally encountered, especially when cultivated upon albumin. We have never seen the branching diphtheria bacilli; and in approximately 9000 blood-serum cultures from cases of diphtheria that have been examined by competent bacteriologists at the laboratory of the Board of Health of Philadelphia, the branching forms of this organism were not observed in a single instance. It is fair to assume, therefore, that this peculiar morphological variation of *bacillus diphtheriæ* is comparatively rare.

On plain nutrient agar-agar (that is, nutrient agar-agar without glycerin); on solidified egg-albumin; on a medium consisting of dried commercial albumin dissolved in bouillon (about 10 grammes of albumin to 100 c.c. of bouillon containing 1 per cent. of grape-sugar); in bouillon without glycerin, and in bouillon to which a bit of hard-boiled egg has been added, the morphology of the organism is about intermediate, in both size and outline, between the forms seen upon glycerin-agar-agar and upon Löffler's blood-serum. There will appear about an equal number of short segmented and longer, irregularly staining forms; but in general the longest are rarely as long as the long forms

seen on blood-serum, and therefore they are not so conspicuous for the irregularity of their staining.

In cultures made upon two sets of nutrient agar-agar tubes, differing only in the fact that one set contains glycerin to the extent of 6 per cent., while the other set contains none, a noticeable difference in morphology can

Bacillus diphtheriæ. A, from a glycerin-agar culture; B, its morphology in a plain agar-agar culture; C, its morphology in blood-serum culture.

usually be made out: while the forms on the glycerin-agar-agar cultures are throughout small, and pretty regular in size, shape, and staining, those on the plain agar-agar are larger, stain less uniformly, vary more in shape, and when stained by Löffler's blue are not so regularly marked by pale transverse lines that give to them the appearance of being made up of numerous short segments.

Though the outline of this organism is more regular under some circumstances than others, it is nevertheless always conspicuous for its manifold variations in shape.

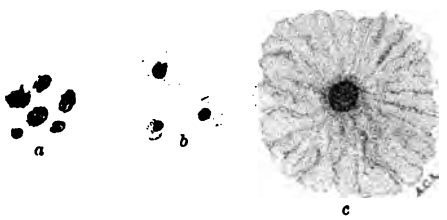
Growth on Serum Mixture.—The medium upon which bacillus diphtheriæ grows most rapidly and luxuriantly, and which is best adapted for determining its presence in diphtheritic exudates, is, as has been stated, the blood-serum mixture of Löffler. (See chapter on Media.) On the blood-serum mixture the colonies of *bacillus diphtheriæ* grow so much more rapidly than the other organisms usually present in secretions and exudations in the throat that at the end of twenty-four hours they are often the only colonies that attract attention ; and if others of similar size are present, they are generally of quite a different aspect. Its colonies are large, round, elevated, grayish-white or yellowish, with a centre more opaque than the slightly irregular periphery. The surface of the colony is at first moist, but after a day or two becomes rather dry in appearance.

A blood-serum tube studded with coalescent or scattered colonies of this organism is so characteristic that one familiar with the appearance can anticipate with tolerable certainty the results of microscopic examination.

Glycerin-agar-agar. — Upon nutrient glycerin-agar-agar the colonies likewise present an appearance that may readily be recognized. They are in every way more delicate in structure than when on the serum mixture. They appear at first, when on the surface, as very flat, almost transparent, dry, non-glistening, round points which are not elevated above the surface upon which they are growing. When slightly magnified they are seen to be granular, and to present

an irregular central marking, which is denser and darker by transmitted light than the thin, delicate zone which surrounds it. As the colony increases in size the thin granular peripheral zone becomes broader, is usually marked by ridges or cracks, and its periphery is notched

FIG. 67.



Colonies of *bacillus diphtheriæ* on glycerin-agar-agar. *a*. Colonies located in the depths of the medium. *b*. Colonies just breaking out upon the surface of the medium. *c*. Fully developed surface-colony.

or scalloped. (Fig. 67, *c*.) These colonies are always quite dry in appearance. When deep down in the agar-agar they are coarsely granular. (Fig. 67, *a*.) They rarely exceed 3 mm. in diameter.

GELATIN.—On gelatin the colonies develop much more slowly than on media that can be retained at a higher temperature. They rarely present their characteristic appearances on gelatin in less than seventy-two hours. They then appear as flat, dry, translucent points, usually round in outline.

When magnified slightly the centre is seen to be more dense than the surrounding zone or zones, for they are sometimes marked by a concentric arrangement of zones. The periphery is irregularly notched. Like the colonies seen on agar-agar, they are granular, but are much more granular when seen in the depths of the gelatin than when on its surface. On gelatin the col-

onies rarely become very large; usually they do not exceed 1.5 mm. in diameter.

Bouillon.—In bouillon it usually grows in fine clumps, which fall to the bottom of the tube, or become deposited on its sides without causing diffuse clouding of the bouillon. Sometimes there are exceptions to this naked-eye appearance: the bouillon may be diffusely clouded; but if one inspect it very closely, particularly if he examine it microscopically as a hanging drop, the arrangement in clumps will always be detected, but the clumps are so small as not to be discernible by the unaided eye.

In bouillon kept at a temperature of 35°–37° C. a soft, whitish pellicle often forms upon the surface.

Changes in reactions of the bouillon. The reaction of the bouillon frequently becomes at first acid, and subsequently again alkaline, changes which can be observed in cultivations in bouillon to which a little rosolic acid has been added. This play of reactions has been attributed to the primary fermentation of the muscle-sugar often present in the bouillon. It does not occur when the medium is free from carbohydrates.

Potato.—On potato at a temperature of 35°–37° C. its growth after several days is invisible, only a thin, dry glaze appearing at the point at which the potato was inoculated. Microscopic examination of scrapings from the potato, after twenty-four hours at 35°–37° C., reveals a decided increase in the number of individual organisms planted.

Stab- and Slant-cultures.—In stab- and slant-cultures on both gelatin and glycerin-agar-agar the surface-growth is seen to predominate over that along the track of the needle in the depths of the media.

Isolated colonies on the surface of either of the media in this method of cultivation present the same characteristics that have been given for the colonies on plates.

The growth in simple stab-cultures does not extend laterally away from beyond the point at which the needle entered the medium.

It is a non-motile organism.

It does not form spores.

It is killed in ten minutes by a temperature of 58° C.

It grows at temperatures ranging from 22° to 37° C., but most luxuriantly at the latter temperature.

Its growth in the presence of oxygen is more active than when this gas is excluded.

STAINING.—In cover-slip preparations made either from the discharges of a diphtheritic patient or from a pure culture of the organism it is seen to stain readily with the ordinary aniline dyes. It stains also by the method of Gram, but the best results are obtained by the use of Löffler's alkaline methylen-blue solution: this brings out the dark points on the pale plasmic body of the bacilli and thus aids in their identification.

For the purpose of demonstrating the Löffler bacilli in sections of diphtheritic membrane, both the Gram method and the fibrin method of Weigert give excellent results.

PATHOGENIC PROPERTIES. When inoculated subcutaneously into the bodies of susceptible animals the result is not the production of septicæmia, as is seen to follow the introduction into animals of certain other organisms with which we shall have to deal, but the bacillus of diphtheria remains localized at the point of inoculation, rarely disseminating further than the nearest lymphatic glands. It develops at the point in

the tissues at which it is deposited, and during its development gives rise to changes in the tissues which result entirely from the absorption of poisonous albumins generated by the bacilli in the course of their development.

In a certain number of cases[1] diphtheria bacilli have been found in the blood and internal organs of individuals dead of the disease; but all that has been learned from careful study of the secondary manifestations of diphtheria tends to the opinion that they are in no way dependent upon the immediate presence of bacteria, and that the occasional appearance of diphtheria bacilli in the internal organs is in all probability accidental, and usually unimportant.

By special methods of inoculation[2] (the injection of fluid cultures into the testicles of guinea-pigs) diphtheria bacilli *can be caused* to appear in the omentum; but this is purely an artificial manifestation of the disease, and one that is probably never encountered in the natural course of events. More rarely similar results follow upon subcutaneous inoculation.

If a very minute portion of either a solid or fluid pure culture of this organism be introduced into the subcutaneous tissues of a guinea-pig or kitten, death of the animal ensues in from twenty-four hours to five days. The usual changes are an extensive local œdema, with more or less hyperæmia and ecchymoses at the

[1] Frosch: "Die Verbreitung des Diphtherie-bacillus in Körper des Menschen," Zeit. für Hygiene und Infektionskrankheiten, 1893, Bd. xiii. pp. 49-52. Booker: Archives of Pediatrics, Aug. 1893. Wright and Stokes: Boston Med. and Surg. Journ., March and April, 1895.

[2] Abbott and Ghriskey: "A Contribution to the Pathology of Experimental Diphtheria," The Johns Hopkins Hospital Bulletin, No. 30, April, 1893.

site of inoculation; swollen and reddened lymphatic glands; increased serous fluid in the peritoneum, pleura, and pericardium; enlarged and hemorrhagic adrenal bodies; occasionally slightly swollen spleen; and sometimes fatty degeneration in the liver, kidney, and myocardium. In guinea-pigs, especially, the liver often shows numerous macroscopic dots and lines on the surface and penetrating the substance of the organ. They vary in size from a pin-point to a pin-head, and may be even larger. They are white and do not project above the surface of the capsule.

The bacilli are always to be found at the site of inoculation, most abundant in the grayish-white, fibrino-purulent exudate. They become fewer at a distance from this, so that the more remote parts of the œdematous tissues do not contain them. They are found not only free, but contained in large number in leucocytes, some of which have fragmented nuclei, or have lost their nuclei. The bacilli within leucocytes, as well as some outside, frequently stain very faintly and irregularly, and may appear disintegrated and dead.

Culture-tubes inoculated from the blood, spleen, liver, kidneys, adrenal bodies, distant lymphatic glands, and serous transudates, generally yield negative results; and negative results are also obtained when these organs are examined microscopically for the bacilli.

Microscopic examination of the tissues at the site of inoculation, as well as of the liver, spleen, kidneys, lymphatic glands, and elsewhere, reveals the presence of localized foci of cell-death, characterized by a peculiar fragmentation of the nuclei of the cells of these parts.

This destruction of nuclei results in the formation

of groups of irregularly shaped, deeply staining bodies, having at times the appearance of particles of dust, while again they may be much larger. Some of them are tolerably regular in outline, while others are irregularly crescentic, dumb-bell, flask-shape, whetstone-shape, or bladder-like in form. Occasionally nuclei having the appearance of being pinched or drawn out can be seen. At some points the fragments are grouped in isolated masses, indicating the location of the nucleus from the destruction of which they originated. These particles always stain much more intensely than do the normal nuclei of the part.[1] Oertel has shown that these peculiar alterations in their distribution are characteristic of human diphtheria, and the demonstration of similar changes in animals inoculated with this organism is important additional proof that diphtheria is caused by it.

By the inoculation of certain animals an affection may be produced in all respects identical with diphtheria as it exists in man. If one open the trachea of a kitten and rub upon the mucous membrane a small portion of a pure culture of this organism, the death of the animal usually ensues in from two to four days. At autopsy the wound will be found covered with a grayish, adherent, necrotic, distinctly diphtheritic layer. Around the wound the subcutaneous tissues will be œdematous. The lymphatic glands at the angle of the jaws will be swollen and reddened. The mucous membrane of the trachea at the point upon

[1] See "The Histological Changes in Experimental Diphtheria," also "The Histological Lesions Produced by the Toxalbumin of Diphtheria," by Welch and Flexner, Johns Hopkins Hospital Bulletin, August, 1891, and March, 1892.

which the bacilli were deposited will be covered with a tolerably firm, grayish-white, loosely attached pseudo-membrane in all respects identical with the croupous membrane observed in the same situation in cases of human diphtheria. In the pseudo-membrane and in the œdematous fluid about the skin-wound *bacillus diphtheriæ* may be found both in cover-slips and in cultures.

From what we have seen—the localization of the bacilli at the point of inoculation, their absence from the internal organs, and the changes brought about in the cellular elements of the internal organs—there is but one interpretation for this process, viz., that it is due to the production of a soluble poison by the bacteria growing at the site of inoculation, which, gaining access to the circulation, produces the changes that we observe in the tissues of the internal viscera.

This poison has been isolated from cultures of *bacillus diphtheriæ*, and is found to belong, not to the crystallizable ptomaïnes, but to the toxic albumins—bodies which, in their chemical composition, are analogous to the poison of certain venomous serpents. By the introduction of this *toxalbumin*, as it is called, into the tissues of guinea-pigs and rabbits the same pathological alterations may be produced that we have seen to follow inoculation with the bacilli themselves, except, perhaps, the production of false membranes.

Under certain circumstances with which we are not acquainted *bacillus diphtheriæ* becomes diminished in virulence or may lose it entirely, so that it is no longer capable of producing death of susceptible animals, and may cause only a transient local reaction from which the animal entirely recovers. Sometimes

this reaction is so slight as to be overlooked, and again careful search may fail to reveal evidence of any reaction at all. This exhibition of the extremes of its pathogenic properties, viz., death of the animal, on the one hand, and only very slight local effects on the other, was at one time thought to indicate the existence of two separate and distinct organisms that were alike in cultural and morphological peculiarities, but which differed in their disease-producing power. Further studies on this point have, however, shown that the genuine *bacillus diphtheriæ* may possess almost all grades of virulence, and that absence of or diminution in virulence can hardly serve to distinguish as separate species those varieties that are otherwise alike; moreover, the histological conditions found at the site of inoculation in animals that have not succumbed, but in which only the local reaction has appeared, are in most cases characterized by the same changes that are seen at autopsy in animals in which inoculation has proved fatal.

In the course of their observations upon a large number of cases Roux and Yersin found that it was not difficult to detect, in the diphtheritic deposits of the same individual, bacilli of identical cultural and morphological peculiarities, but of very different degrees of virulence, and that with the progress of the disease toward recovery the less virulent varieties often became quite frequent.[1]

There is, moreover, a mild form of diphtheria, etio-

[1] It must not be assumed from this that the bacilli lose their virulence entirely, or that they *all* become attenuated with the establishment of convalescence, for this is contrary to what experience has shown to be the case.

logically speaking, affecting only the mucous membrane of the nares, known as membranous rhinitis, from which it is very common to obtain cultures in all respects identical with those from typical diphtheria, save for their inability to *kill* susceptible animals. On inoculation these cultures produce only local reactions, but these are characterized histologically by the same kind of tissue-changes that follow inoculation with the fully virulent organism.

Clinically, membranous rhinitis is never such an alarming disease as is laryngeal or pharyngeal diphtheria, and, as stated, the organisms causing it are often of a low degree of virulence, though they are, nevertheless, genuine diphtheria bacilli.

For those organisms that are in all respects identical with the virulent *bacillus diphtheriæ*, save for their inability to kill guinea-pigs, the designation "pseudo-diphtheritic bacillus" is usually employed; but from such observations as those just cited we are inclined to the opinion that *pseudo*-diphtheritic, as applied to an organism in all respects identical with the genuine bacillus, except that it is not *fatal* to susceptible animals, is a misnomer, and that it would be more nearly correct to designate this organism as the attenuated or non-virulent diphtheritic bacillus, reserving the term "pseudo-diphtheritic" for that organism or group of organisms (for there are probably several) that is enough like the diphtheria bacillus to attract attention, but is distinguishable from it by certain morphological and cultural peculiarities aside from the question of virulence.

It is a well-known fact that many pathogenic organisms—conspicuous among these being *micrococcus lanceolatus, staphylococcus pyogenes aureus, streptococcus*

pyogenes, and the group of so-called "hemorrhagic septicæmia" organisms—undergo marked variations in their pathogenic properties; and yet these organisms, when found either devoid of this peculiarity, or possessing it in a diminished degree, are not designated as "pseudo" forms, but simply as the organisms themselves, the virulence of which, from various causes, has been modified.

It must nevertheless be admitted that in the course of microscopic examination of materials from various sources, including the pharynx, one occasionally encounters micro-organisms whose morphology is so like that of the genuine *bacillus diphtheriæ* as to create suspicion, and yet they are at the same time sufficiently unlike it to make one cautious in forming an opinion as to their real nature. In the routine examination of such large numbers of cases as are submitted for diagnosis to municipal laboratories it is not practicable to subject all specimens to the crucial animal-test, and for this reason there can be no doubt that the results of microscopic examination alone were formerly occasionally incorrect. Through the suggestion of Neisser[1] we are fortunately enabled to overcome this difficulty in very large part. He has found that by the use of a particular staining method the appearance of *bacillus diphtheriæ* is strikingly unlike that of the confusing forms. His differential method comprehends the following manipulations: the culture to be tested should be grown upon Löffler's blood-serum mixture solidified at 100° C.; it should develop at a temperature not lower than 34° C. and not higher

[1] Neisser: Zeitschrift für Hygiene und Infektionskrankheiten, 1897, Bd. xxiv.

than 36° C.; and it should be not younger than nine and not older than twenty-four hours. A cover-glass preparation made from such a culture is stained as follows:

a. It is subjected to the following mixture for from one to three seconds:

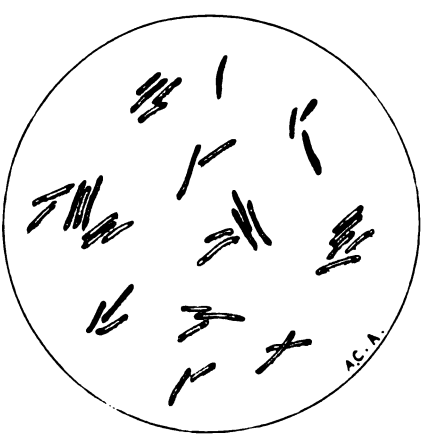
Methylene-blue (Grübler's)	1 gramme.
Alcohol (96 per cent.)	20 c.c.

When dissolved, mix with

Acetic acid	50 c.c.
Distilled water	950 c.c.

b. After thoroughly rinsing in water, it is stained for from three to five seconds in vesuvin (Bismarck-brown),

FIG. 68.



Bacillus diphtheriæ, stained by Neisser's method.

2 grammes, dissolved in 1 litre of boiling distilled water, filtered, and allowed to cool. It is again rinsed in water

and examined as a water-mount, or it may be dried and mounted in balsam.

When so treated the diphtheria bacillus appears as faintly stained brown rods, in which from one to three dark-blue granules are always to be observed. The dark granules are at one or both poles of the cell, are more or less oval, and usually seem to bulge a little beyond the contour of the bacillus in which they are located. (See Fig. 68.) From Neisser's observations and those of others,[1] as well as from personal experience, it seems safe in the vast majority of cases to regard all bacilli that do not stain in the way described as distinct from *bacillus diphtheriæ*.[2]

NOTE.—Prepare cover-slip preparations from the mouth-cavities of healthy individuals and from those having decayed teeth. Do they correspond in any way with those made from diphtheria? Do the same with different forms of sore-throat. Do the peculiarities of any of the organisms suggest those of the bacillus of diphtheria? Wherein is the difference?

In cultures and cover-slips made from both diphtheritic and from innocent sore-throats are any organisms almost constantly present? Which are they, and what are their characteristics?

Which are the predominating organisms in the anginas of scarlet fever?

Do these organisms simulate, in their cultural and morphological peculiarities, any of the different species with which you have been working?

[1] Fränkel: Berliner klin. Wochenschrift, 1897, No. 50.

[2] Bergey: Publications of the University of Pennsylvania, New Series, No. 4, 1898.

Do the diphtheria bacilli disappear from the throat with the disappearance of the membrane? How long do they persist? When obtained from the throats of convalescents are they still pathogenic for guinea-pigs?

Prepare a bouillon culture of virulent bacillus diphtheriæ; after it has been growing for thirty-six hours at 37°–30° C. inoculate a guinea-pig subcutaneously with about 0.1 c.c. of it. If the animal dies, note carefully the findings at autopsy, especially the distribution of the bacilli. Now add to this culture sufficient pure carbolic acid or trikresol to kill all bacteria in it, and inject under the skin of another guinea-pig varying amounts of the culture so treated, beginning with 0.05 c.c.; determine the minimum fatal dose, and note in which respects the post-mortem findings simulate and in which they differ from those of the first animal. Should any of the animals survive the injections of the disinfected culture, note carefully their condition from day to day, particularly any fluctuations in weight. When they have quite recovered inoculate them with living, virulent diphtheria bacilli. Do the results correspond with those obtained with guinea-pigs that have never been treated at all? Explain the results.

CHAPTER XXI.

Typhoid fever—Study of the organism concerned in its production—*Bacterium coli commune*—Its resemblance to the bacillus of typhoid fever—Its morphological, cultural, and pathogenic properties—Its differentiation from *bacillus typhosus*—Methods of isolating the typhoid bacillus.

BACILLUS TYPHOSUS.

THE organism which was discovered in the tissues of typhoid cadavers microscopically by Eberth, and subsequently isolated in pure culture and described by Gaffky, is now generally recognized as the etiological

FIG. 69.

Bacillus typhosus, from culture twenty-four hours old, on agar-agar.

FIG. 70.

Bacillus typhosus, showing flagella stained by Löffler's method.

factor in the production of typhoid fever. It may be described as follows:

It is a bacillus about three times as long as broad, with rounded ends. It may appear at one time as very short ovals, at another time as long threads, and both

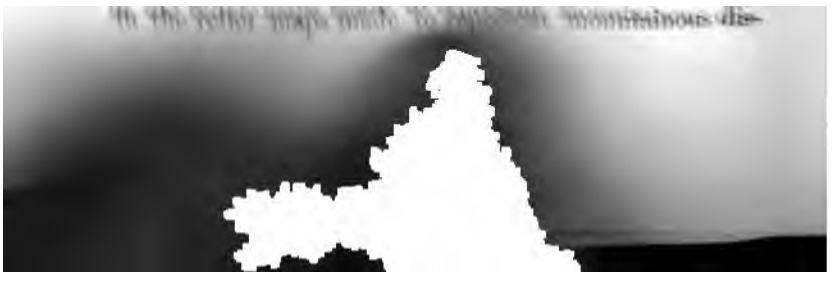
[redacted]

... its breadth remains toler- ... morphology presents little that ... Fig. 69. It stains a ... the aniline dyes than do most of ... actively motile, and ... method of Löffler (see ... staining is seen to possess ... organs in the form of fine, ... in large numbers to all parts ... These flagella are not ..., nor are they rendered ... staining.

... typhoid fever the organ- ... in the blood, urine, and ... the tissues of the spleen, liver, ... gastric glands, and intestines.

... —Its growth, when seen in the depths of the medium, presents nothing characteristic, appearing simply as round or oval, finely granular points. On the surface it develops as very superficial, blue-white colonies, with irregular borders. They are a little denser at the centre than at the periphery.

... folds, ... seen



tricts. (Fig. 71.) These colonies have sometimes the appearance of flattened pellicles of glass-wool, and usually a pearl-like lustre.

On AGAR-AGAR the colonies present nothing typical.

STAB-CULTURES. — In stab-cultures the growth is mostly on the surface, there being only a very limited development down the track made by the needle. The surface-growth has the same appearance in general as that given for the colonies.

POTATO.—The growth on potato is usually described as luxuriant but invisible, making its presence evident only by the production of a slight increase of moisture at the inoculated point, and by a limited resistance offered to a needle when it is scraped across the track of growth. While this is so in many cases, yet it cannot be considered as invariable, for at times this organism develops more or less visibly on potato.

POTATO-GELATIN.—The growth is similar to that upon ordinary nutrient gelatin.

MILK.—It does not cause coagulation when grown in sterilized milk.

BOUILLON.—It causes uniform clouding of the bouillon and brings about a slightly acid reaction.

INDOL FORMATION.—It is customary to regard this organism as devoid of the power of forming indol; in fact, this has hitherto been considered one of its important differential peculiarities. By the usual methods of cultivation and testing the indol reaction is not observed in cultures of the typhoid bacillus. It has recently been shown, however, by Peckham, that by repeated transplantation, at short intervals, into either Dunham's peptone solution, or, preferably, a freshly prepared alkali-tryptone solution, made from trypton-

ized beef-muscle, that the indol-producing function may be induced in the genuine typhoid bacillus obtained directly from the spleens of typhoid cadavers.[1]

It does not produce gaseous fermentation. On lactose-litmus-agar-agar it grows as pale-blue colonies, causing no reddening of the surrounding medium; though if glucose be substituted for lactose, both the colonies and the surrounding medium may become red. In the fermentation-tube, in glucose or lactose bouillon, no evolution of gas as a result of fermentation occurs.

It does not form spores. The irregularities of staining so commonly seen in this organism have in some instances led to the belief that the pale, unstained portions of the bacilli indicate the presence of spores. More reliable tests, however, have demonstrated the error of this opinion. (What is the most trustworthy test of spore-formation?)

It grows at any temperature between 20° and 38° C., though more favorably at the latter point. It is very sensitive to high temperatures, being killed by an exposure of ten minutes to 60° C., and in a much shorter time to slightly higher temperatures.

It does not liquefy gelatin.

It grows both with and without oxygen.

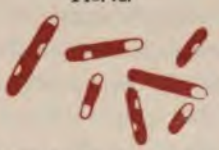
It does not grow rapidly.

Owing to a tendency to retraction of its protoplasm from the cell-envelope and the consequent production of vacuoles in the bacilli, the staining of this organism is frequently more or less irregular. At some points in a single cell marked differences in the intensity of the

[1] A. W. Peckham: "The Influence of Environment Upon the Biological Functions of the Colon Group of Bacilli," Journal of Experimental Medicine, 1897, vol. ii.

staining will be seen, and here and there areas quite free from color can commonly be detected. These colorless portions are often so sharply defined that they

FIG. 72.



Diagrammatic representation of retraction of protoplasm, with production of pale points, in *bacillus typhosus.*

look as if they had been punched out with a sharp instrument. (See Fig. 72.)

PRESENCE IN TISSUES.—It is not easy to demonstrate this organism in tissues unless it is present in large numbers. The manipulations to which the sections are subjected in being mounted often rob the bacilli of their stain, and render them invisible, or nearly so. If, however, sections be stained in the carbol-fuchsin solution, either at the ordinary temperature of the room or at a higher temperature (40° to 45° C.), then washed in absolute alcohol, and cleared in xylol and mounted in balsam, the bacilli (particularly if the tissue be the liver and spleen) can readily be detected, massed together in their characteristic clumps. If used in the same way, the alkaline methylene-blue solution gives also very satisfactory results.

In searching for the typhoid bacilli in tissues this peculiar deposition in clumps must always be borne in mind, otherwise much labor will be expended in vain. In tissues the typhoid bacilli do not lie scattered about in the same way as do the organisms in tissues from certain other conditions—septicæmia, for instance; they

are not of necessity distributed along the course of the capillaries, but are localized in small clumps through the organs, and it is for these clumps, which are easily detected under a low-power objective, that one should search. This peculiar clumping of the typhoid bacilli in the tissues cannot be satisfactorily explained. It may possibly be due to the specific clumping or agglutinating influence that typhoid blood has been shown to have upon the typhoid bacillus, a phenomenon that is readily demonstrable in the test-tube or under the microscope. In other words, may it not be simply the result of an intracapillary "Widal reaction"? (See Widal Reaction.)

When the section is prepared for examination, if it be gone over with a low-power objective, one will notice at irregular intervals little masses that look in every respect like particles of staining-matter which have been precipitated upon the section at that point. When these masses are examined with a higher power objective they will be found to consist of small ovals or short rods so closely packed that the individuals composing the clump can often be seen only at the extreme periphery of the mass. This is the characteristic appearance of the typhoid organism in tissues. The little masses are usually in the neighborhood of a capillary.

Result of Inoculation into Lower Animals.—A great many experiments have been made in a variety of ways with the view of reproducing the pathological conditions of this disease, as seen in man, in the tissues of lower animals, but with practically no success. From the time of its discovery up to within a comparatively recent date there was an almost continuous controversy concerning the infective properties of *bacillus*

typhosus for animals. By some it was held that the effects of its introduction into animals were manifestly of toxic[1] origin, while others regarded them as evidences of genuine infection.[2] These diversities of opinion are hardly surprising when we remember that animals never suffer naturally from a disease similar to typhoid fever, and therefore offer many obstacles to its faithful reproduction, and that the vigor of this organism when cultivated from various sources is liable to a wide range of fluctuation. For a time there seemed to be good grounds for the opinion that under exceptional circumstances *bacillus typhosus* did exhibit truly infective properties, and the reported experiments of Cygnæus[3] in particular, as well as a single observation by the writer,[4] in no wise weakened this opinion. By a variety of methods Cygnæus demonstrated that this organism possessed the property of multiplying within the internal organs of animals and of causing constitutional symptoms and pathological lesions that very closely simulated those of typhoid fever as seen in man. In 1890 the writer called attention to the lesions found in one of a number of rabbits that had succumbed to intravenous injection of large amounts of fluid cultures of this organism.

In this case there was an ulcer in the ileum which was macroscopically and microscopically identical with those found at autopsy in the small intestine of human

[1] Toxic—poisonous results not necessarily accompanied by the growth of organisms throughout the tissues.

[2] Infective or septic—poisoning of the tissues as a result of the growth of bacteria within them.

[3] Cygnæus: Ziegler's Beiträge zur Anat. und Path., 1890, Bd. vii. Heft 3, S. 377.

[4] Bulletin of the Johns Hopkins Hospital, 1890, vol. i. p. 63.

subjects dead of this disease. The typhoid bacilli were not only obtained from the spleen of the animal by culture method, but the characteristic clumps were also demonstrated microscopically in sections of the organ.

It must be said, however, that such results are extremely rare. As a rule, the only effects that follow the introduction of this organism into animals are referable to the intoxicating action of the materials used. In fact, the results of modern investigations have placed *bacillus typhosus* in the category of toxin-producers, and through the use of the toxins produced by it animals have been rendered immune from otherwise fatal doses. The serum of such animals has also been shown to possess a certain degree of immunizing power.[1]

In connection with the inoculation of animals with *bacillus typhosus* observations of a most important nature have been made by Sanarelli[2] upon the artificial induction of susceptibility to its pathogenic action. He found that rabbits, guinea-pigs, and mice could be rendered susceptible to infection by this organism by preliminary injections into them of the products of growth of certain saprophytes — *proteus vulgaris*, *bacillus prodigiosus*, and *bacterium coli commune*; and that by whatever means the animal was subsequently inoculated with fresh cultures of the typhoid bacillus, either into the circulation or into the peritoneal cavity, death resulted in from twelve to forty-eight hours, with the pathological alterations most conspicuous in the digestive tract, and particularly in the small intestine. In these cases the infection is general, and the organisms

[1] Pfeiffer and Kolle: Zeitschrift für Hygiene und Infektionskrankheiten, 1896, Bd. xxi. S. 208.

[2] Sanarelli: Annales de l'Institut Pasteur, 1892, tome vi.

may be recovered from the blood and internal organs. It is the opinion of Sanarelli that the toxic conditions produced by the preliminary injections of the products of growth of the saprophytic organisms may be considered analogous to a similar condition that may occur in man from the absorption of abnormal products of fermentation from the intestinal canal—an auto-intoxication that so reduces the resistance of the individual as to render him susceptible to infection by the bacillus of typhoid fever, should it gain access to his alimentary tract.

More recently it was reported by Alessi[1] that rats, guinea-pigs, and rabbits, when compelled to breathe the gaseous products of decomposition from the contents of a cesspool, or from other decomposing matters, gradually became susceptible to infection by the typhoid bacillus; but unfortunately for the value of this observation the description given by Alessi of the two cultures of so-called typhoid bacilli used by him for inoculation was in one case certainly not that of the typhoid organism, and in the other the culture used had been kept under artificial conditions so long as hardly to be reliable for tests of this character.

The importance of these observations in their bearing upon the etiology of typhoid fever, if they are demonstrated by subsequent experiment to be trustworthy, is too obvious to necessitate emphasis, and it is greatly to be desired that they may not be permitted to pass unnoticed, but that others interested may find occasion to institute experiments in the same direction, with the hope that some light may be shed upon the mooted

[1] Alessi: Centralblatt für Bakteriologie un. Parasitenkunde 1894, Bd. xv. No. 7, p. 228.

question concerning the influence of gaseous products of decomposition upon the health of individuals, and particularly upon the part played by them in diminishing natural resistance to infection.[1]

Because of the variations in the morphology and cultural peculiarities of this organism, and because of the difficulty experienced in efforts to reproduce in lower animals the conditions found in the human subject, typhoid fever is bacteriologically one of the most unsatisfactory of the infectious diseases.

A number of other organisms appear botanically to be closely related to the typhoid bacillus, and with our present methods for studying them they so closely simulate it that the difficulty of identifying this organism is sometimes very great. In addition the variability constantly seen in pure cultures of the typhoid bacillus itself in no way renders the task more simple.

For example, the morphology of the typhoid bacillus is conspicuously inconstant; its growth on potato, which was formerly described as characteristic, may, with the same stock, at one time be the typical invisible development, at another time it may grow in a way easily to be seen with the naked eye; and the change of reaction which it is said to produce in bouillon is sometimes much more intense than at others. The indol-producing function, hitherto regarded as absent from this organism, is now known to be occasionally demonstrable by ordinary methods, and frequently by special methods of cultivation. (Peckham, *l. c.*) The

[1] See paper by the author: "The Effects of the Gaseous Products of Decomposition upon the Health, and Resistance to Infection, of Certain Animals that are Forced to Respire Them." Transactions of the Association of American Physicians, 1895, vol. x. pp. 16-44.

only properties possessed by it that may be said to be constant are its motility; its inability to cause gaseous fermentation of glucose, lactose, or saccharose; its incapacity for coagulating milk; and its growth on gelatin plates; but there are other bacilli which possess these same characteristics to a degree that renders their differentiation from the typhoid organism often a matter that requires the careful application of all the different tests.

An interesting reaction of the typhoid bacillus is seen when it is brought in contact with the blood-serum from human beings sick of typhoid fever, or from animals that have survived inoculation with cultures of this organism. This reaction consists of a peculiar alteration in the relation of the organisms to one another in the fluid. As ordinarily seen in a hanging drop of bouillon, the typhoid bacilli appear as single, actively motile cells; when to such a drop a drop of dilute serum from a case of typhoid fever is added the motility of the organism gradually becomes lessened, and finally ceases, and the bacteria congregate in larger and smaller clumps. The reaction may also be produced in another way, viz., by adding to about 4 or 5 c.c. of a twenty-four-hour-old bouillon culture of typhoid bacilli in a narrow test-tube about eight drops of serum from a case of typhoid fever, after which the tube is placed in the incubator. After a few hours the normally clouded culture is seen to have undergone a change; instead of the diffuse cloud caused by the growth, the fluid is found clear and contains within it flocculent masses of the bacteria that have agglutinated together as a result of the specific action of the serum used.

For the hanging-drop test sufficient serum may be obtained from a needle-prick in the finger, while for the test-tube reaction a larger amount is needed; this may be obtained from blood drawn from a superficial vein by means of a hypodermic syringe, or from the cleansed skin by a wet-cup, or, better still, from a small cantharides blister.

It is proper to state, however, that occasionally cultures of genuine typhoid bacilli are encountered that do not respond to this peculiar influence of typhoid blood, even though the blood be tested at different stages of the disease, and even though it causes the characteristic cessation of motion and clumping with other cultures of this organism upon which it may be tried.

When employed conversely—*i. e.*, for deciding if the serum used is from a case of typhoid fever or not—the reaction constitutes "Widal's serum diagnosis of typhoid fever." For this purpose it is often necessary to test several cultures of genuine typhoid bacilli, from different sources and of varying degrees of vitality, before a culture is procured that gives the reaction most conspicuously and quickly with genuine typhoid serum. This culture is then to be set aside, to be used for this test with serums from doubtful cases of the disease.

Widal's Reaction with Dried Blood.—For clinical purposes it is of importance to know that this reaction can be obtained from dried blood—*i. e.*, by the method suggested by Wyatt Johnston, of Montreal. In this method a drop of the blood to be tested, obtained by a needle-prick in the cleansed finger or lobe of the ear, is collected on a bit of clean, unglazed paper and allowed to dry. The paper is then folded, kept free from contamination, and taken to the labora-

tory. With a medium-size platinum-wire loop a drop of sterile bouillon, water, or physiological salt-solution is gently rubbed upon the drop of dried blood until the contents of the loop are of a dark amber color; this is then mixed with a drop of a bouillon culture of typhoid bacilli on a cover-glass, which is mounted upon the hollow-ground slide as a hanging drop, when the effect of the diluted blood upon the culture can be observed with the microscope. The reaction, if positive, should occur within a half hour. Many object to this method because it is impossible accurately to dilute the blood by the plan used. A number of tests have shown us that preparations made in this way correspond roughly with a fresh-blood dilution of from 1 : 15 to 1 : 20, as determined by the hæmoglobinometer. In a small number of cases in which parallel tests were made with this and with fresh fluid serum the results were concordant. We are inclined to the opinion, however, that in doubtful cases, in which all the available clinical evidence is opposed to either the positive or negative results of the test, the difficulty is much more certainly cleared away by the use of highly diluted and exactly diluted fresh serum than by this method. Competent observers are of the opinion that in all such cases the quantity of serum in the hanging drop should be decreased until it is present in the proportion of from (not less than) 1 : 50 to 1 : 60, and that, if after exposure to this dilution for two hours the bacilli are still motile and not clumped together, or the reaction is deficient in only one or the other of these peculiarities, the case from which the serum was obtained may be safely regarded as not typhoid fever.

Experience with the dry-blood method at the Mu-

nicipal Laboratory of Philadelphia in more than 12,000 examinations from about 10,000 febrile conditions, leads us to regard the culture used as one of the most important factors in the test. After deciding upon the most suitable culture for the reaction—and it is often necessary to try a great number from various sources—we have adopted the plan of daily transplanting the culture into fresh bouillon and keeping it at a temperature rarely above 20°–22° C. The bacilli grown under these circumstances are usually somewhat longer than when cultivated at higher temperature, and they exhibit a regular, gliding motility that renders it more easy to follow the individual cells under the microscope than when they possess the usual active, darting motion.

In the group of cases examined by us by the dry-blood method, including typhoid and other febrile conditions, there is a discrepancy between the clinical and the laboratory diagnosis in from 2 to 3 per cent. of the cases examined.

In the hands of all who have carefully employed the Widal reaction for the diagnosis of typhoid fever the results are reported to have been almost uniformly satisfactory. In the great majority of cases the reaction is, so far as experience indicates, specific—*i. e.*, a typical reaction does not occur between typhoid serum and organisms other than the typhoid bacillus, nor between the typhoid bacillus and serums other than those of typhoid fever. There are, however, confusing reactions—so-called pseudo-reactions—in which more or less clumping of the bacilli and a diminution of motion, without complete cessation, are observed. These reactions have been seen to occur with normal blood and

with blood from other febrile conditions. It is said by Johnston and McTaggart[1] that they can be prevented if cultures of *just the proper degree of vitality* are employed; and this corresponds with the results of a fairly wide personal experience with the test.

The blood of certain animals, as well as a number of chemical substances, such as corrosive sublimate, alcohol, salicylic acid, resorcin, and safranin in high dilution, cause agglutination of the typhoid bacilli; but the reaction is not specific, for in most cases they have the same effect on other motile bacilli.

The method is yet in the experimental stage, and there are still numerous features not entirely clear. In the light of present experience, however, it is fair presumptive evidence that the serum is from a case of typhoid fever when unmistakable agglutination and cessation of motion are seen in from fifteen to twenty minutes after typhoid bacilli are mixed with the serum of a suspicious febrile condition.

All the preceding points should be borne in mind in the examination of drinking-water supposed to be contaminated by typhoid dejections, for the organisms which most closely approach the typhoid bacillus in growth and morphology are just those organisms which would appear in water contaminated from cesspools—*i. e.*, the organisms constantly found in the normal intestinal tract. Even in the stools of typhoid-fever patients the presence of these normal inhabitants of the intestinal tract renders the isolation of the typhoid organisms somewhat troublesome.

METHODS OF ISOLATING THE TYPHOID BACILLUS. —*Bacillus typhosus* is so variable in many of its bio-

[1] Johnston and McTaggart: Montreal Medical Journal, March, 1897.

logical peculiarities, and is so closely simulated in certain respects by a group of other organisms to which it appears to be botanically related, that its identification, especially outside the infected body, is usually a matter of considerable difficulty and uncertainty. For these reasons many efforts have been made to discover specific reactions for the organism, and with this end in view many methods have been devised for its isolation from water, fæces, sewage, and other matters believed to contain it. None of them, however, has given general satisfaction, and many have proved wholly untrustworthy. Those worthy of some degree of confidence are as follows:

Elsner's method. The medium employed is an acid mixture of gelatin, potato-juice, and potassium iodide. It contains neither peptone nor sodium chloride. On this medium it is claimed that the ordinary, rapidly growing, liquefying saprophytes do not develop, and that the colon bacillus and typhoid bacillus find it favorable for growth. These are differentiated from one another by the macroscopic and microscopic character of their colonies—*i. e.*, the growth of the colon colonies differs little or not at all from that seen on ordinary nutrient gelatin, while that of the typhoid colonies is so slow that they are scarcely visible at the end of twenty-four hours. After forty-eight hours the latter appear under the low power of the microscope as small, pale, finely granular, almost transparent bodies easily distinguished from the coarser, brownish colonies of the colon bacillus.

While the method is useful, it has its limitations, and is not always reliable. At times colon colonies develop in a manner that may readily cause one to

mistake them for typhoid colonies; while typhoid colonies may present the characteristics of those due to the growth of the colon bacillus. This is especially the case in plates more than forty-eight hours old that have been kept at ordinary room-temperature.

In our experience the most serviceable feature of this method is the elimination of many of the common saprophytes usually present in mixtures containing typhoid and colon bacilli. The majority of them do not grow upon gelatin made by this method, which will now be described.

The description given by Elsner[1] of the mode of preparation of the medium is so incomplete and unsatisfactory in most of the important details that practically all who use the method have been obliged to develop their own technique from the general suggestions made in his original communication. The "Elsner medium" that has given satisfaction in our hands is prepared as follows: grate one kilogramme of peeled potatoes and allow to stand over night in a refrigerator. Then press out all juice, using an ordinary meat-press for the purpose. Filter this fresh juice *cold*, to remove as much of the starch-granules as possible; if this is not done, the starch when heated swells to such an extent as to render filtration almost impracticable. Boil the filtrate and filter again. Test the filtrate for acidity by titrating 10 c.c. with a decinormal solution of sodium hydroxide, the indicator used being 6 drops of the ordinary $\frac{1}{2}$ per cent. solution of phenolphtalein in 50 per cent. alcohol. The acidity of the juice should be such as to require 3 c.c. of a deci-

[1] Elsner: Zeitschrift für Hygiene und Infektionskrankheiten, 1896, Bd. xxi. S. 25.

normal sodium hydroxide solution to neutralize 10 c.c. of the juice. (Potter.) If the acidity is found to be greater than this, which is usually the case, dilute with water until the proper degree is reached. If less than this, the juice may be concentrated by evaporation. It is desirable that this acidity should be due to the acids normally present in the potato, and that it should not be artificially obtained by the addition of other acids. Now add 10 per cent. of gelatin (no peptone and no sodium chloride), dissolve by boiling, and again test the acidity, using 10 c.c. of the mixture and phenolphtalein as before. Deduct 3 c.c. (the acidity of the potato-juice, that is to be maintained) from the number of c.c. of the decinormal sodium hydroxide solution required to neutralize the 10 c.c. of the gelatin mixture, and from the resulting figure calculate the amount of *normal* solution of sodium hydroxide needed for the entire volume, and add it. Boil, clarify with an egg, and filter through paper in the usual manner. To the filtrate add potassium iodide in the proportion of 1 per cent. Decant into tubes and sterilize.

Stoddart's method. The principle upon which this method is based may be briefly stated as follows: if the typhoid bacillus be cultivated in a *semi-solid* nutrient medium at about 35° C., its growth is not circumscribed or confined to the immediate neighborhood of the point of inoculation; whereas if a less actively motile organism—the colon bacillus, for example—be cultivated under similar circumstances there is no apparent tendency for it to diffuse itself through the medium; its development is confined to the point of inoculation. In the former case the medium becomes clouded throughout; in the latter it remains clear, except at the immediate,

sharply defined seat of growth. If a mixture of the two organisms be taken upon the point of a needle and deposited at a single spot upon a plate made from a semi-solid nutrient medium, or be planted as a stab-culture in a test-tube of the same medium, the growth of the colon bacillus remains about the part touched, or along the track of the needle, while that of the typhoid bacillus spreads as a delicate cloud for some distance beyond. The medium recommended by Stoddart for the test is a mixture of equal parts of ordinary meat-infusion—peptone-agar-agar (1 per cent. agar-agar) and meat-infusion-peptone-gelatin (10 per cent. gelatin). In this mixture the proportion of agar-agar present is, obviously, 0.5 per cent., and that of the gelatin 5 per cent. These amounts are sufficient to keep it just about solid when exposed to 35° C.

A limited experience with the method leads us to regard it as a useful addition to our means for the isolation and identification of the typhoid and typhoid-like bacilli. It is not infallible in its indications.

It is employed in two ways—for plates and for tube cultures, though the former is perhaps the more useful. Here the liquefied culture mixture is poured into a sterile Petri dish and allowed to gelatinize; after which it is inoculated at a single point, or at several points, with the pure culture, or with the mixture of organisms to be tested, and placed at 35° C. The characteristics of the growths of the typhoid and of the colon bacillus are given above.[1]

Hiss's method. A short time subsequent to Stoddart's publication Hiss called attention to a similar application

[1] For further details, see Stoddart: Journal of Pathology and Bacteriology, 1897, vol. iv. p. 429.

of a technical procedure, which in principle, with some important additions, is almost identical with that of Stoddart. In this method also advantage is taken of the fact that in semi-solid nutrient media the greater motility of the typhoid bacillus enables it to diffuse more readily through the medium than can the less active colon bacillus. The endeavor of Hiss was to discover a method whereby this peculiarity would be favored, or at least not checked, in the typhoid, and more or less suppressed in the colon bacillus. A series of experiments demonstrated that if peptone be omitted and glucose be added to the semi-solid medium, the absence of the former important nutritive substance and the excess of acidity resulting from the fermentation of the latter had only slight effect upon the characteristic development of the typhoid bacillus (a diffuse clouding of the medium), while the influence upon the growth of the colon bacilli was to prevent, in many cases, their tendency to cloud the medium by sharply restricting their growth to the point at which they were deposited.[1]

The composition of the medium used is:

Agar-agar	5 grammes.
Gelatin	80 "
Liebig's beef-extract	5 "
Sodium chloride	5 "
Glucose	5–10 "
Water	1000 c.c.

The gelatin should be added after the agar-agar and other ingredients are dissolved; the volume of the mass is then brought to 1000 c.c., and finally the reaction is corrected. This should be equivalent to a degree of acidity that would require 15 c.c. of a normal sodium

[1] Hiss: Journal of Experimental Medicine, 1897, vol. ii. No. 6, p. 677.

hydroxide solution to the litre to bring it to the phenolphtalein neutral point.

When planted as stab-cultures in this medium and kept at body-temperature the growth of *bacillus typhosus* appears simply as a diffuse cloud, with little or no tendency to appear more concentrated along the track of the needle; while the development of the colon bacillus is confined to the neighborhood of the needle-track, is moderately dense, is accompanied by the formation of gas-bubbles, and the surrounding gelatin is more or less clear.

These distinctions were found by Hiss to be much more marked with the slowly or feebly motile specimens of the colon bacillus than when the actively motile varieties were used. Many of these latter, except for their power to ferment glucose with liberation of gas, were almost indistinguishable from the typhoid bacillus in so far as their power to wander through and cloud the medium was concerned. For the isolation and differentiation of colonies of the two organisms by the plate method the following medium was employed:

Agar-agar	10 grammes.
Gelatin	25 "
Liebig's beef-extract	5 "
Sodium chloride	5 "
Glucose	10 "
Water	1000 c.c.

The reaction of this medium is equivalent to 2 per cent. of normal acid to the litre—*i. e.*, an acidity that would require 20 c.c. of normal sodium hydroxide solution to the litre to bring it to the phenolphtalein neutral point. In plates made from this medium the deep colonies of *bacillus typhosus* are small, more or less spherical, and

have a rough, irregular outline. Their most characteristic feature "consists of well-defined, filamentous outgrowths, ranging from a single thread to a complete fringe around the colony. The young colonies are at times composed solely of threads." The fringing threads grow almost straight out from the colonies. The surface colonies are small and have usually a dense centre that is surrounded by an almost transparent zone or by a fringe of threads somewhat similar to those seen about the deeper colonies.

The deep colonies of the colon bacillus are, as a rule, larger, denser, of an oval or lens-shape, and are more sharply circumscribed than those of *bacillus typhosus*. On the surface they are also larger, and, as a rule, spread out as a moderately thick layer from a denser centre. The younger the colonies of the typhoid bacillus the more characteristic their appearance. They are seen at their best after from 16 to 18 hours' growth at 37.5° C.[1]

Method of Capaldi and Proskauer.[2] As a result of an elaborate series of experiments, these authors recommend the use of two special culture-media for the differentiation of the typhoid and colon bacilli.

Medium No. 1 consists of:

Asparagine	0.2	per cent.	in distilled water.
Mannite	0.2	"	"
Sodium chloride	0.02	"	"
Magnesium sulphate	0.01	"	"
Calcium chloride	0.02	"	"
Mono-potassium phosphate	0.2	"	"

[1] The reader is referred to the original article for many important details that are not included here.

[2] Capaldi and Proskauer: Zeitschrift für Hygiene und Infektionskrankheiten, 1896, Band xxiii. S. 452.

Medium No. 2 consists of:

Witte's peptone	2.0 per cent. in distilled water.
Mannite	0.1 " "

Both media are to be sterilized, the reaction brought to the litmus *neutral* point with caustic potash solution, and enough litmus tincture then added to them to cause a distinct, though not too intense, purple color. After this they are to be again sterilized, when they are ready for use.

After 20 hours at 37°–38° C. typical colon bacilli and the varieties of this organism develop in both solutions, but produce *acid only in medium No. 1.*

The typhoid bacillus grows *only* in medium *No. 2*, and its growth is accompanied by the production of an *acid* reaction.

The growth of bacillus coli in medium No. 2 is accompanied by a slight *alkaline* reaction.

The alterations in reaction are indicated by the corresponding changes in the color of the litmus tincture in the media.

It is interesting to note that in this test the usual reactions produced by these organisms in peptone media containing the ordinary fermentable carbohydrates, such as glucose and lactose, are reversed.

The authors state that this method has thus far shown itself to be infallible for the differentiation of cultures of typhoid and colon bacilli obtained by them from every available source.

The spleen of a patient dead of typhoid fever is the most reliable source from which to obtain cultures of the typhoid bacillus for study. But it must always be remembered that the same channels through which the

typhoid bacillus gains access to this viscus are likewise open to other organisms present in the intestines, and for this reason *bacterium coli commune*, a normal inhabitant of the colon, may also be found in this locality.

NOTE.—Obtain a pure culture of typhoid bacilli, and from this make inoculations upon a series of potatoes of different ages and from different sources. Do they all grow alike?

Before sterilizing render another lot of potatoes slightly acid with a few drops of very dilute acetic acid; render others very slightly alkaline with dilute caustic soda. Are any differences in the growths noticeable?

Make a series of twelve tubes of peptone solution to which rosolic acid has been added. Inoculate them all with as nearly the same amount of material as possible (one loopful from a bouillon culture into each tube); place them all in the incubator. Is the color-change, as compared with that of the control-tube, the same in all cases.

Compare the morphology of cultures of the same age on gelatin, agar-agar, and potato.

Select a culture in which the vacuolations are quite marked. Examine this culture unstained. Do the organisms look as if they contained spores? How would you demonstrate that the vacuolations are not spores? What is the crucial test for spores?

Obtain from normal fæces a pure culture of the commonest organism present. Write a full description of it. Now make parallel cultures of this organism and of the typhoid bacillus on all the different media. How do they differ? In what respects are they similar?

BACTERIUM COLI COMMUNE; BACILLUS COLI COMMUNIS COLON BACILLUS; BACILLUS NEAPOLITANUS OF EMMERICH.

This organism was discovered by Escherich, in 1885, in the intestinal discharges of milk-fed infants. It has since been demonstrated to be a normal inhabitant of the intestines of man and of certain domestic animals (bovines, hogs, dogs).

For a time after its discovery it was considered of but little importance and attracted attention only because of its resemblance, in certain respects, to the bacillus of typhoid fever, with which it was occasionally confounded. In this particular it still serves as a subject for study. Some have even gone so far as to regard them as but varieties of one and the same species, though in the present state of our knowledge this is an assumption for which as yet there are not sufficient grounds. That they possess in common certain general points of resemblance and often approach one another in some of their biological peculiarities is true; but, as we shall learn, they each possess peculiarities which, when considered together, render their differentiation from one another a matter of but little difficulty.

With the wider application of bacteriological methods to the study of pathological processes it was occasionally observed that, under favorable circumstances, *bacillus coli communis* disseminated from its normal habitat and appeared in remote organs, often associated with diseased conditions. This was at first considered of but little importance, and its presence in these localities was usually regarded as accidental. Its

repeated appearance, however, in different parts of the body outside of the intestines, and the frequency of its association with pathological conditions, ultimately attracted attention to it, and in consequence during the past few years a great deal has been written concerning the possible pathogenic nature of this organism.

The fact that it is a commensal species, always intimately associated with certain of our life-processes, together with the fact that it is known to appear in organs other than that in which it is normally located, and that its occurrence in diseased conditions is not rare, justifies the opinion that it is one of the most important of the micro-organisms with which we have to deal.

While not generally considered a pathogenic organism, there is, nevertheless, sufficient evidence to warrant the statement that, under favorable conditions, with which we are not entirely familiar, this organism may assume pathogenic properties, so that its presence in diseased conditions is not always to be considered as accidental, though this is frequently the case.

The morphological and cultural peculiarities of *bacterium coli commune* are as follows:

Morphology.—In shape it is a rod with rounded ends, sometimes so short as to appear almost spherical, while again it is seen as very much longer threads. Often both forms are associated in the same culture. It may occur as single cells, or as pairs joined end to end.

It has no peculiar morphological features that can aid in its identification, for in this respect it simulates many other organisms. It is usually said to be motile, and undoubtedly is motile in the majority of

cases; but its movements are at time so sluggish that a positive opinion is often difficult.

By Löffler's method of staining, flagella can be demonstrated, though usually not in such numbers as are seen to occur on the typhoid fever bacillus.

It does not form spores.

It grows both with and without free oxygen.

On Gelatin.—On the surface its colonies appear as small, dry, irregular, flat, blue-white points that are commonly somewhat dentated or notched at the margin. They are a trifle denser at the centre than at the periphery, and are often marked at or near the middle by an oval or round nucleus-like mass—the original colony from which the layer on the surface developed. When located in the depths of the gelatin, and examined with a low-power lens, they are at first seen to be finely granular and of a very pale greenish-yellow color; later they become denser, darker, and much more markedly granular; in shape they are round, oval, and lozenge-like. When the surface colonies are viewed under a low power of the microscope they present essentially the same appearance as that given for the colonies of the bacillus of typhoid fever, viz., they resemble flattened pellicles of glass-wool, or patches of finely ground colorless glass. Colonies of this organism on gelatin are frequently encountered that cannot be distinguished from those resulting from the growth of *bacillus typhosus;* though, as a rule, their growth is a little more luxuriant.

In stab- and smear-cultures on gelatin the surface-growth is flat, dry, and blue-white or pearl color. Limited growth occurs along the track of the needle in the depths of the gelatin. As the culture becomes

older the gelatin round about the surface-growth may gradually lose its transparency and become cloudy, often quite opaque. In still older cultures small root- or branch-like projections from the surface-growth into the gelatin are sometimes seen. At times these may be of a distinctly crystalline appearance.

It does not cause liquefaction of gelatin.

Its growth on nutrient agar-agar and on blood-serum is luxuriant, but not characteristic.

In bouillon it causes diffuse clouding with sedimentation. In some bouillon cultures an attempt at pellicle-formation on the surface may be seen, but this is exceptional. In old bouillon cultures the reaction becomes alkaline and a decided fæcal odor may be detected.

Its growth on potato is rapid and voluminous, appearing after twenty-four to thirty-six hours in the incubator as a more or less lobulated layer of a drab, dark-cream, or brownish-yellow color.

In neutral milk containing a little litmus tincture the blue color is changed to red after from eighteen to twenty-four hours in the incubator, and, in addition, the majority of cultures cause firm coagulation of the casein in about thirty-six hours, though frequently this takes longer. *Very rarely* the litmus may indicate the production of acid and no coagulation occur.

In media containing glucose it grows rapidly and causes active fermentation, with liberation of carbonic acid and hydrogen. If cultivated in solid media to which glucose (2 per cent.) has been added, the gas-formation is recognized by the appearance of numerous bubbles along and about the points of growth. If cultivated in fluid media, also containing glucose, in the

fermentation-tube, evidence of fermentation is given by the collection of gas in the closed arm of the tube.

On lactose-litmus-agar-agar its colonies are pink and the color of the surrounding medium is changed from blue to red.

It produces indol in both bouillon and peptone solution.

In Dunham's peptone solution it produces indol in from forty-eight to seventy-two hours.

It stains with the ordinary aniline dyes. It is decolorized when treated by the method of Gram.

By comparing what has been said of *bacillus typhosus* and of *bacterium coli commune* it will be seen that while they simulate each other in certain respects, they nevertheless possess individual characteristics by which they may readily be differentiated. The least variable of the differential points are:

1. Motility of *bacillus typhosus* is much more conspicuous, as a rule, than is that of *bacterium coli commune*.

2. On gelatin colonies of the typhoid bacillus develop more slowly than do those of the colon bacillus.

3. On potato the growth of the typhoid bacillus is usually invisible (though not always); while that of the colon bacillus is rapid, luxuriant, and always visible.

4. The typhoid bacillus does not cause coagulation of milk with acid reaction. The colon bacillus does this in from thirty-six to forty-eight hours in the incubator.

5. The typhoid bacillus never causes fermentation, with liberation of gas, in media containing glucose, lactose, or saccharose. The colon bacillus is conspicuous for its power of causing gaseous fermentation in such solutions.

6. In nutrient agar-agar or gelatin containing lactose and litmus tincture, and of a slightly alkaline reaction, the color of the colonies of typhoid bacillus is pale blue, and there is no reddening of the surrounding medium; while colonies of the colon bacillus are pink and the medium round about them becomes red.

7. The typhoid bacillus does not, *as a rule*, possess the property of producing indol in solutions of peptone; the growth of the colon bacillus in these solutions is accompanied by the production of indol in from forty-eight to seventy-two hours at 37° to 38° C.

Animal Inoculations.—As with the bacillus of typhoid fever, the results of inoculation of animals with cultures of this organism cannot be safely predicted. According to the observations of Escherich, Emmerich, Weisser, and others, the effects that do appear are in most instances to be attributed to the toxic rather than to the infective properties of the culture used.

When introduced into the subcutaneous tissues of mice it has no effect, while similar inoculations of guinea-pigs are sometimes (not always) followed by abscess-formation at the point of operation, or by alterations very similar to those produced by intravascular inoculation, viz., death in less than twenty-four hours, accompanied by redness of the peritoneum and marked hyperæmia and ecchymoses of the small intestine, together with swelling of Peyer's patches. The cæcum and colon may remain unchanged or present enlarged follicles. There may or may not be an accumulation of fluid in the abdominal cavity; but peritonitis is rarely present. The small intestine may contain bloody mucus.

Intravenous inoculation of rabbits may be followed by similar changes, with often the occurrence of diar-

rhœa before death, which may, in the acute cases, result in from three to forty hours. In another group of cases acute fatal intoxication does not result, and the animal lives for weeks or months, dying ultimately of what appears to be the effects of a slow or chronic form of infection. For a few hours after inoculation these animals present no marked symptoms; exceptionally, somnolence and diarrhœa have been observed at this period, indicating acute intoxication from which the animal has recovered. The affection is unattended by fever. The most marked symptom is loss of weight. This is usually progressive from the first or second day after inoculation, with slight fluctuations until death.

At autopsy the animal is found to be emaciated. The subcutaneous tissues and the muscles appear pale and dry. The serous cavities, particularly the pericardial, may contain an excess of serum. The viscera are anæmic. The spleen is small, thin, and pale. Exceptionally ulcers and ecchymoses are observed in the cæcum, but generally there are no lesions of the intestinal tract.

The most striking and constant lesions, those most characteristic of the affection, are in the bile and in the liver; in some cases the quantity of bile may not exceed the normal, but in others the gall-bladder may be abnormally distended with bile. The bile is nearly colorless or has a pale yellowish or brownish tint, with little or no greenish color. Its consistence is much less viscid than normal, being often thin and watery. It usually contains small, opaque, yellowish particles or clumps which can be seen floating in it, even through the walls of the gall-bladder. These clumps consist microscopically of bile-stained, apparently necrotic, epithelial

cells; leucocytes in small numbers; amorphous masses of bile-pigment, and bacteria often in zoöglœa-like clumps. Similar material is found in the larger bile-ducts.

The liver frequently contains opaque, whitish or yellowish-white spots and streaks of irregular size and shape, which give a peculiar mottling to the organ when present in large number. These areas may be numerous, or only one or two may be found. In size they range from minute points to areas of from 2 to 3 cm. in extent. By microscopic examination they are found to represent localities where the liver-cells have undergone necrosis accompanied by emigration of leucocytes, and the cells about them are in a condition of fatty degeneration. In sections of the liver masses of the bacilli may be discovered in and about the necrotic foci just described.

At these autopsies the colon bacillus is not found generally distributed through the body, but is only to be detected in the bile, liver, and occasionally in the spleen.[1]

[1] Consult paper by Blachstein on this subject, Johns Hopkins Hospital Bulletin, 1891, vol. ii., p. 96.

CHAPTER XXII.

The spirillum (comma bacillus) of Asiatic cholera—Its morphological and cultural peculiarities—Pathogenic properties—The bacteriological diagnosis of Asiatic cholera.

At the conference held in Berlin in 1884 for the purpose of discussing Asiatic cholera from the sanitary aspect, it was announced by Koch[1] that he had discovered in the intestinal evacuations of individuals suffering from Asiatic cholera a micro-organism that he believed to be the cause of the malady. The importance of this statement necessarily attracted widespread attention to the subject, and as one of the consequences there existed, for a short time following, some skepticism as to the accuracy of Koch's claim. These doubts arose as a result of a series of contributions from other observers, who endeavored to prove that the organism found by Koch in cholera evacuations was common to other localities, and was not a specific accompaniment of this disease. It was not very long, however, before it was evident that these objections were based upon untrustworthy observations, and that by reliable methods of investigation the organism to which he had called attention could be easily differentiated from each of those with which it was claimed to be identical.

This organism, known both as the spirillum of Asiatic cholera, and, because of its morphology, as Koch's

[1] Verhandlungen der Conferenz zur Erörterung der Cholerafrage, 1884, Berlin.

"comma bacillus," is identified by the following peculiarities:

SPIRILLUM CHOLERÆ ASIATICÆ.

MORPHOLOGY.—It is a slightly curved rod, ranging from about 0.8 to 2 μ in length and from 0.3 to 0.4 μ in thickness—that is to say, it is usually from about one-half to two-thirds the length of the tubercle bacillus, but is thicker and plumper. Its curve is frequently not more marked than that of a comma, and, indeed, it is often almost straight; at times, though, the curve is much more pronounced, and may even describe a semicircle. Occasionally the curve may be double, one comma joining another, with their convexities pointing in opposite directions, so that a figure similar to the letter S is produced. In cultures long spiral or undulating threads may often be seen. From these appearances this organism cannot be considered as a bacillus, but rather as an intermediate type between the bacilli and the spirilla. Koch thinks it not improbable that the short comma forms represent segments of a true spirillum, the normal form of the organism. (Fig. 73.)

It does not form spores, and we have no reliable evidence that it possesses the property of entering, at any time, a stage in which its powers of resistance to detrimental agencies are increased.

It is a flagellated organism, but has only a single flagellum attached to one of its ends.

It is actively motile, especially in the comma stage; though the long spiral forms also possess this property.

GROUPING.—As found in the slimy flakes in the intestinal discharges from cholera patients, Koch likens its [illegible] of grouping to that seen in a school of small fish

when swimming up stream—*i. e.*, they all point in nearly the same direction, and lie in irregularly parallel, linear

FIG. 73.

Spirillum of Asiatic cholera. Impression cover-slip from a colony thirty-four hours old.

groups that are formed by one comma being behind the other without being attached to it.

FIG. 74.

Involution-forms of the spirillum of Asiatic cholera, as seen in old cultures.

On cover-slip preparations made from cultures in the ordinary way there is nothing characteristic about the grouping; but in impression cover-slips made from young cultures the short commas will nearly always be seen in small groups of three or four, lying together in such a way as to have their long axes nearly parallel to one another. (See Fig. 73.)

In old cultures in which development has ceased it undergoes degenerative changes, and the characteristic

comma and spiral shapes may entirely disappear, their place being taken by irregular involution-forms that present every variety of outline. (See Fig. 74.) In this stage they take on the stain very feebly, and often not at all.

Cultural Peculiarities.—On plates of nutrient gelatin that have been prepared from a pure culture of this organism and kept at a temperature of from 20° to 22° C., development can often be observed after as short a period as twelve hours, but frequently not before sixteen to eighteen hours. This is especially true of the first or "original" plate, containing the largest number of colonies. At this time the plate will present to the naked eye an appearance that has been likened to a ground-glass surface, or to a surface that has been stippled with a finely pointed needle, or one upon which very fine dust has been sprinkled. This appearance is due to the presence of minute colonies closely packed together upon the surface of the gelatin. In the depth of the gelatin can also be seen closely packed, small points, likewise representing growing colonies. As growth progresses liquefaction occurs around the superficial colonies, and in consequence this plate is usually entirely liquid after from twenty-four to thirty hours; the developmental phases through which the colonies pass cannot, therefore, be studied upon it.

On plates 2 and 3, where the colonies are more widely separated, they can be seen after twenty-four to thirty hours as small, round or oval, white or cream-white points, and when located superficially a narrow transparent zone of liquefaction can be detected around them. As growth continues this liquefaction extends downward rather than laterally, and the colony ulti-

mately assumes the appearance of a dense, white mass lying at the bottom of a sharply-cut pit or funnel containing transparent fluid. This liquefaction is never very widespread nor rapid, and rarely extends more than one millimetre beyond the colony proper. On plates containing few colonies there is little or no tendency for them to become confluent, and they rarely exceed 2 to 3 mm. in diameter.

FIG. 75.

Developmental phases of colonies of the spirillum of Asiatic cholera at 20° to 22° C. on gelatin. × about 75 diameters.

a. After sixteen to eighteen hours. *b*. After twenty-four to twenty-six hours. *c*. After thirty-eight to forty hours. *d*. After forty-eight to fifty hours. *e*. After sixty-four to seventy hours.

When examined under a low magnifying lens the very young colonies (sixteen to eighteen hours old) appear as pale, translucent, granular globules of a very delicate greenish or yellowish-green color, sharply outlined, and not perfectly round. (See *a*, Fig. 75.) As growth progresses this homogeneous granular appearance is replaced by an irregular lobulation, and ultimately the sharply-cut margin of the colony becomes dentated or scalloped. (See *b* and *c*, Fig. 75.) After forty-eight hours (and frequently sooner) liquefaction of the gelatin

has taken place to such an extent that the appearance of the colony is entirely altered. Under a magnifying glass the colony proper is now seen to be ragged about its edges, while here and there shreds of the colony can be detected scattered through the liquid into which it is sinking. These shreds evidently represent portions of the colony that became detached from its margin as it gradually sank into the liquefied area.

At *d*, in Fig. 75, is seen a representation of the several appearances afforded by the colonies at this stage. At the end of the second, or during the early part of the third day, the sinking of the colonies into the liquefied pits resulting from their growth is about complete, and under a low-power lens they now appear as dense, granular masses, surrounded by an area of liquefaction through which can be seen granular prolongations of the colony, usually extending irregularly between the periphery and the central mass. (See *e*, Fig. 75.) If the periphery be examined, it will be seen to be fringed with delicate, cilia-like lines that radiate from it in much the same way that cilia radiate from the ends of the columnar epithelial cells lining the air-passages.

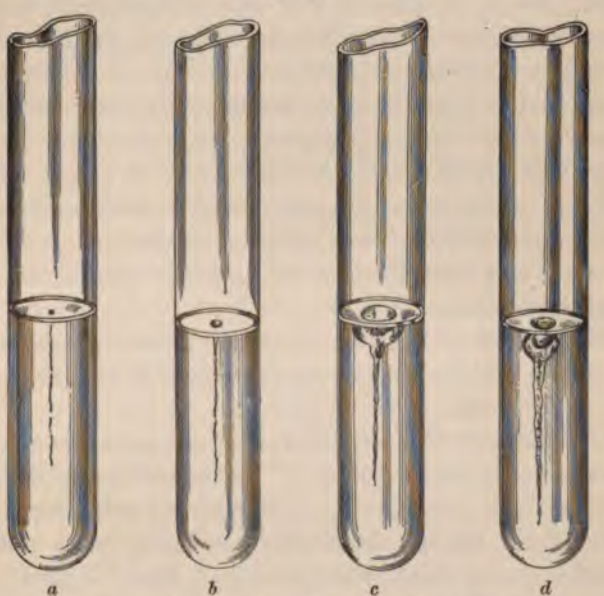
These are the more marked phases through which the colonies of this organism pass in their development on gelatin plates. In some cultures the various phases here given pass in succession more quickly, while in cultures from other sources they may be somewhat retarded.

On plates of nutrient agar-agar the appearance of the colonies is not characteristic. They appear as round or oval patches of growth that are moist and moderately transparent. The colonies on this medium at 37° C.

naturally grow to a larger size than do those upon gelatin at 22° C.

In stab-cultures in gelatin there appears at the top of the needle-track after thirty-six to forty-eight hours at 22° C. a small, funnel-shaped depression. As the growth progresses liquefaction occurs about this point. In the centre of the depression can be distinguished

FIG. 76.



Stab-culture of the spirillum of Asiatic cholera in gelatin, at 18° to 20° C.

a. After twenty-four hours. *b*. After forty-eight hours. *c*. After seventy-two hours. *d*. After ninety-six hours.

a small, dense, whitish clump, the colony itself. As growth continues the depression increases in extent and ultimately assumes an appearance that consists in

the apparent sinking of the liquefied portion in such a way as to leave a perceptible air-space between the top of the liquid and the surface of the solid gelatin. The growth now appears to be capped by a small air-bubble. The impression given by it at this stage is not only that there has been a liquefaction, but also a coincident evaporation of the fluid from the liquefied area and a constriction of the superficial opening of the funnel. (See *a*, *b*, *c*, and *d*, Fig. 76.) Liquefaction is not especially active along the deeper portions of the track made by the needle, though in stab-cultures in gelatin the liquefaction is much more extensive than that usually seen around colonies on plates. It spreads laterally at the upper portion, and after about a week a large part of the gelatin in the tube may have become fluid, and the growth will have lost its characteristic appearance.

Stab- and smear-cultures on agar-agar present nothing characteristic.

Its growth in bouillon is luxuriant, causing a diffuse clouding and the ultimate production of a delicate film upon the surface.

In sterilized milk of a neutral or amphoteric reaction at a temperature of 36° to 38° C. it develops actively, and gradually produces an acid reaction, with coagulation of the casein. It retains its vitality under these conditions for about three weeks or more. The blue color of milk to which neutral litmus tincture has been added is changed to pink after thirty-six or forty-eight hours at body-temperature.

Its growth in peptone solution, either that of Dunham (see Special Media) or the one preferred by Koch, viz., 2 parts of Witte's peptone, 1 part of sodium chloride, and 100 parts of distilled water, is accompanied by

the production of both indol and nitrites, so that after eight to twelve hours in the incubator at 37° C. the rose color characteristic of indol appears upon the addition of sulphuric acid alone. (See Indol Reaction.)

(What does the presence of nitrites in these cultures signify?)

In peptone solution to which rosolic acid has been added the red color is very much intensified after four or five days at 37° C.

Its growth on potato of a slightly acid reaction is seen after three or four days at 37° C. as a dull, whitish, non-glistening patch at and about the site of inoculation. It is not elevated above the surface of the potato, and can only be distinctly seen when held to the light in a particular position. Growth on acid potato occurs, however, only at or near the body-temperature, owing probably to the acid reaction, which is sufficient to prevent development at a lower temperature, but does not have this effect when the temperature is more favorable. On solidified blood-serum growth is usually said to be accompanied by slow liquefaction. I have not succeeded in obtaining this result on Löffler's serum, nor have I detected anything characteristic about its growth on this medium.

The temperature most favorable for its growth is between 35° and 38° C. It grows, but more slowly, at 17° C. Below 16° C. no growth is visible.

It is not destroyed by freezing. When exposed to 65° C. its vitality is destroyed in five minutes.

It is strictly aërobic, its development ceasing if the supply of oxygen be cut off.

It does not grow in an atmosphere of carbonic acid, but is not killed by a temporary exposure to this gas.

It does not grow in acid media, but flourishes best in media of neutral or slightly alkaline reaction. It is so sensitive to the action of acids that at 22° C. its development is arrested when an acid reaction equivalent to 0.066 to 0.08 per cent. of hydrochloric or nitric acid is present. (Kitasato.)

Under artificial cultivation the maximum development of this organism is reached in a comparatively short time; after this it remains quiescent for a period, and finally degeneration begins. The dying comma bacilli become altered in appearance and assume the condition known as "involution-forms." (See Fig. 74.) When in this state they take up coloring-reagents very faintly or not at all, and may lose entirely their characteristic shape.

When present with other bacteria, under conditions favorable to growth, the comma bacillus at first grows much more rapidly than do the others; in twenty-four hours it will often so outnumber the other organisms present that microscopic examination might lead one to regard the material under consideration as a pure culture of this organism. Its conspicuous development under these circumstances does not, however, last longer than two or three days; degeneration and death begin, and the other organisms gain the ascendency. This fact has been taken advantage of by Schottelius[1] in the following method devised by him for the bacteriological examination of dejections from cholera patients:

In dejections not examined immediately after being passed it is often difficult, because of the large number of other bacteria that may be present, to detect

[1] Deutsche med. Wochenschrift, 1885, No. 14.

with certainty the cholera organism by microscopic examination. It is advantageous in these cases to mix the dejections with about double their volume of slightly alkaline beef-tea, and allow them to stand for about twelve hours at a temperature between 30° and 40° C. There appears at the end of this time, *especially upon the surface of the fluid*, a conspicuous increase in the number of comma bacilli, and cover-slip preparations made from the upper layers of the fluid will reveal an almost pure culture of this organism.

It is not improbable that a similar process occurs in the intestines of those suffering from Asiatic cholera, viz., a rapid multiplication of the comma bacilli that have gained access to the intestines takes place, but lasts for only a short time, when the comma bacilli begin to disappear, and after a few days their place is taken by other organisms.

In connection with his experiments upon the poison produced by the cholera organism Pfeiffer[1] states that in very young cultures, grown under access of oxygen, there is present a body that possesses intensely toxic properties. This primary cholera-poison stands in very close relation to the material composing the bodies of the bacteria themselves, and is probably an integral constituent of them, for the vitality of the cholera spirilla can be destroyed by means of chloroform or thymol, and by drying, without apparently any alteration of this poisonous body. Absolute alcohol, concentrated solutions of neutral salts, and a temperature of 100° C., decompose this substance, leaving intact secondary poisons which possess a similar physiological activity, but only when given in from ten to

[1] Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xi. S. 393.

twenty times the dose necessary to produce the same effects with the primary poison.

Other members of the vibrio family also, namely, *vibrio Metchnikovi* and that of Finkler and Prior (see description of these species), contain, according to Pfeiffer, closely related poisons.

Experiments upon Animals.—As a result of experiments for the purpose of determining if the disease can be produced in any of the lower animals it has been found that white mice, monkeys, cats, dogs, poultry, and many other animals are not susceptible to infection by the methods usually employed in inoculation experiments. When animals are fed on pure cultures of the comma bacillus no effect is produced, and the organisms cannot be obtained from the stomach or intestines. They are destroyed in the stomach, and do not reach the intestines; they are not demonstrable in the fæces of these animals. Intravascular injections of a pure culture into rabbits are followed by an illness, from which the animals usually recover in from two to three days; intraperitoneal injections into white mice are, as a rule, followed by death in from twenty-four to forty-eight hours; the conditions in both instances most probably resulting from the toxic activities of the poisonous products of growth of the organisms present in the culture employed. None of the lower animals has ever been known to suffer spontaneously from Asiatic cholera.

The failure to induce cholera in animals by feeding, or by injection of cultures into the stomach, was shown by Nicati and Rietsch[1] to be due to the destructive action of the acid gastric juice on the bacilli. They

[1] Archiv. de Phys. norm. et path., 1885, t. vi., 3e sér. Comptes rendus, xcix. p. 928. Revue de Hygiène, 1885. Revue de Médecine, 1885, v.

showed that if cultures of this organism were introduced into the alimentary tract of certain animals in such a manner that they would not be subjected to the influence of the gastric juice, a condition pathologically closely simulating cholera as it occurs in man could be produced. For this purpose the common bile-duct was ligated, after which the cultures were injected directly into the duodenum. Such interference with the flow of bile lessens intestinal peristalsis, and thus permits development of the bacilli at the point at which they are deposited—that is, the portion of the intestine having an alkaline reaction and beyond the influence of the acid stomach-juice.

By this method Nicati and Rietsch, Van Ermengem,[1] Koch,[2] and others were enabled to produce in the animals upon which they operated a condition that was, if not identical, at all events very similar pathologically to that seen in the intestines of subjects dead of the disease.

At a subsequent conference held in Berlin in 1885 Koch[3] described the following method, by means of which he had been able to obtain a much greater degree of constancy in all his efforts to produce cholera in lower animals: bearing in mind the point made by Nicati and Rietsch as to the effect produced by the acid reaction of the gastric juice, this reaction was first to be neutralized by injecting through a soft catheter passed down the œsophagus into the stomach 5 c.c. of a 5 per cent. solution of sodium carbonate. Ten or fifteen minutes later this was to be followed by the injection into the stomach (also through a soft catheter) of 10 c.c. of a

[1] Recherches sur le Microbe du Choléra Asiatique, Paris-Bruxelles, 1885. Bull. de l'Acad. roy. de Méd. de Belgique, xviii., 3e sér.

[2] Loc. cit.

[3] Ibid., 1885.

bouillon culture of the cholera spirillum. For the purpose of arresting peristalsis and permitting the bacteria to remain in the stomach and upper part of the duodenum for as long a time as possible, the animal was to receive, immediately following the injection of the culture, an intraperitoneal injection, by means of a hypodermic syringe, of 1 c.c. of tincture of opium for each 200 grammes of its body-weight. Shortly after this last injection deep narcosis sets in and lasts from a half to one hour, after which the animal is as lively as ever. Of 35 guinea-pigs inoculated in this way by Koch, 30 died of an affection that was, in general, very similar to Asiatic cholera as seen in man.

The condition of those animals before death is described as follows: twenty-four hours after the operation the animal appears unwell; there is loss of appetite, and the animal remains quiet in its cage. On the following day a paralytic condition of the hind extremities appears, which, as the day wears on, becomes more pronounced; the animal lies quite flat upon its abdomen or on its side, with legs extended; respiration is weak and prolonged, and the pulsations of the heart are hardly perceptible; the head and extremities are cold, and the body-temperature is frequently subnormal. The animal usually dies after remaining in this condition for a few hours.

At autopsy the small intestine is found deeply injected and filled with flocculent, colorless fluid. The stomach and intestines do not contain solid masses, but fluid; when diarrhœa does not occur, firm scybala may be detected in the rectum. Both by microscopic examination and by culture methods comma bacilli are found present in the small intestine in practically pure culture.

More recently Pfeiffer[1] has determined that essentially similar constitutional effects may be produced in guinea-pigs by the intraperitoneal injection of relatively large numbers of this organism. His plan is to scrape from the surface of a fresh culture on agar-agar as much of the growth as can be held upon a medium size wire loop. This is then finely divided in 1 c.c. of bouillon, and by means of a hypodermic syringe is injected directly into the peritoneal cavity. When virulent cultures have been used this operation is quickly followed by a fall in the temperature of the animal that is gradual and continuous until death ensues, which usually occurs in from eighteen to twenty-four hours after the operation, though exceptionally the animal recovers, even after having exhibited marked symptoms of profound toxæmia.

Continuing his studies upon this disease, Pfeiffer[2] has demonstrated that it is possible to render an animal tolerant to or immune from the poisonous properties of this organism by repeated injections of non-fatal doses of dead cultures (cultures that have been killed by the vapor of chloroform or by heat). He also demonstrated that animals so immunized possess a specific germicidal action toward the cholera spirillum—*i. e.*, if into the peritoneal cavity of an animal immunized from Asiatic cholera living cholera spirilla be introduced, they will all be destroyed (disintegrated) within a relatively short time. Furthermore, if the serum of an animal immunized from cholera be injected into the peritoneal cavity of another animal of the same species, but not so

[1] Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xi. and xiv.
[2] Ibid., 1894, Bd. xvii. S. 355; 1894, Bd. xviii. S. 1; 1895, Bd. xx. S. 197.

protected, and immediately afterward living cholera spirilla be introduced, a similar disintegration and destruction of the bacteria will also result. He shows that a more or less definite relation exists between the amount of serum and the number of organisms introduced. Such a destruction of the comma bacillus by the serum of an immunized animal does not occur outside the animal body—that is, it cannot be demonstrated in a test-tube. The specificity of this reaction is suggested by Pfeiffer as a means of differentiating the cholera spirillum from other suspicious species, for no such bacteriolytic action is observed if species other than the cholera spirillum be introduced into the peritoneal cavity of animals immunized from Asiatic cholera.

Pfeiffer has further demonstrated that the serum of animals artificially immunized from Asiatic cholera has an agglutinating effect upon fluid cultures of the cholera spirillum similar to that seen when typhoid bacilli are mixed with serum from typhoid cases, or from animals artificially immunized from typhoid infection or intoxication. (See Agglutinin.)

General Considerations.—In all cases of Asiatic cholera, and *only* in this disease, the organism just described can be detected in the intestinal evacuations. The more acute the case and the more promptly the examination is made after the evacuations have passed from the patient, the less difficulty will be experienced in detecting the organism.

In some cases the organism can be detected in the vomited matters, though by no means so constantly as in the intestinal contents.

As a rule, bacteriological examination fails to reveal the presence of the organisms in the blood and internal

organs in this disease, though Nicati and Rietsch claim to have obtained them from the common bile-duct in rapidly fatal cases, and in two out of five cases they were present in the gall-bladder. Doyen and Rasstschewsky[1] found them in the liver in pure culture, and Tizzoni and Cattani[2] in both the blood and the gall-bladder.

The cholera spirillum is a facultative parasite; that is to say, it apparently finds in certain parts of the world, particularly in those countries in which Asiatic cholera is endemic, conditions that are not entirely unfavorable to its development outside of the body. This has been found to be the case not only by Koch, who detected the presence of the organism in water-tanks in India, but by many other observers who have succeeded in demonstrating its growth under conditions not embraced in the ordinary methods employed for the cultivation of bacteria.[3]

The results of experiments having for their object the determination of the length of time during which this organism may retain its vitality in water are conspicuous for their irregularity. In the transactions of the congress in Berlin for the discussion of the cholera question, it is stated, in connection with this point, that the experiments made with tank-water in India sometimes resulted in demonstrating the multiplication of the organisms introduced into it, while in other cases they died very quickly.

[1] Reference to Vratch, 1885, in Allg. Med. Central Zeitung, Berlin.

[2] Centralblatt für die med. Wissenschaften, 1886, No. 43.

[3] Obviously all pathogenic bacteria that have been isolated under artificial methods of cultivation are *facultative* parasites. Were they *obligate* parasites their cultivation upon dead materials would be impossible.

On February 8, 1884, comma bacilli were found in a tank at Saheb-Began, in Calcutta, and it was possible to demonstrate them in a living condition up to February 23d.

Koch states that in ordinary spring-water or well-water the organisms retained their vitality for thirty days, whereas in the canal-water (sewage) of Berlin they died after six or seven days; but if this latter were mixed with fæcal matters, the organisms retained their vitality for but twenty-seven hours; and in the undiluted contents of cesspools it was impossible to demonstrate them after twelve hours. In the experiments of Nicati and Rietsch they retained their vitality in sterilized distilled water for twenty days; in Marseilles canal-water (sewage), for thirty-eight days; in sea-water, sixty-four days; in harbor-water, eighty-one days; and in bilge-water, thirty-two days.

In the experiments of Hochstetter, on the other hand, they died in distilled water in less than twenty-four hours in five of seven experiments; in one of the two remaining experiments they were alive after a day, and in the other after seven days.

In one test with the water-supply of Berlin the organism retained its vitality for 267 days, and in another for 382 days, notwithstanding the fact that many other organisms were present at the same time. There is no ready explanation for these variations, for they depend apparently upon a number of factors which may act singly or together. For example, in general it may be said that the higher the temperature of the water in which these organisms are present, up to 20° C., the longer do they retain their vitality; the purer the water—that is, the poorer in organic

matters—the more quickly do the organisms die, whereas the richer it is in organic matter the longer do they retain their vitality.

Still another point that must be considered in this connection is the antagonistic influences under which they find themselves when placed in water containing large numbers of organisms that are, so to speak, at home in water—the so-called normal water-saprophytes.

The effect of light upon growing bacteria must not be lost sight of, for it has been shown that a surprisingly large number of these organisms are robbed of their vitality by a relatively short exposure to the rays of the sun; and it is therefore not unlikely that the non-observance of this fact may be, in part at least, accountable for some of the discrepancies that appear in the results of these experiments.

In his studies upon the behavior of pathogenic and other micro-organisms in the soil Carl Fränkel[1] found that the cholera spirillum was not markedly susceptible to those deleterious influences that cause the death of a number of other pathogenic organisms. During the months of August, September, and October cultures of the comma bacillus that had been buried in the ground at a depth of three metres retained their vitality; on the other hand, in other months, particularly from April to July, they lost their vitality when buried to the depth of only two metres. At a depth of one and a half metres vitality was not destroyed, and there was a regular development in cultures so placed.

As a result of experiments performed in the Imperial Health Bureau at Berlin, it was found that the bodies of guinea-pigs that had died of cholera induced by

[1] Zeitschrift für Hygiene, Bd. ii. S. 521.

Koch's method of inoculation contained no living cholera spirilla when exhumed after having been buried for nineteen days in wooden boxes, or for twelve days in zinc boxes. In a few that had been buried in moist earth, without having been encased in boxes, when exhumed after two or three months, the results of examinations for cholera spirilla were likewise negative.

Kitasato,[1] in his experiments with the cholera organism, found that when mixed with the intestinal evacuations of human beings under ordinary conditions it lost its vitality in from a day and a half to three days. If the evacuations were sterilized before the cultures were mixed with them, the organisms retained their vitality from twenty to twenty-five days. He was unable to come to any definite conclusion as to the cause of these phenomena.

It was demonstrated by Hesse[2] and by Celli[3] that many substances commonly employed as food-stuffs serve as favorable materials for the development of the cholera organisms. In his experiments upon its behavior in milk Kitasato[4] found that at a temperature of 36° C. the cholera spirillum developed very rapidly during the first three or four hours, and outnumbered the other organisms commonly found in milk. They then diminished in number from hour to hour as the acidity of the milk increased, until finally their vitality was lost; at the same time the common saprophytic bacteria increased in number. Relatively the same process occurs at a lower temperature, from 22° to 25°

[1] Zeitschrift für Hygiene, Bd. v. S. 487.
[2] Ibid., Bd. v. S. 527.
[3] Bollettino della R. Acad. Med. di Roma, 1888.
[4] Zeitschrift für Hygiene, Bd. v. S. 491.

C.; but it is slower, the maximum development of the cholera organisms being reached at about the fifteenth hour, after which time they were outnumbered by the ordinary saprophytes present.

From the foregoing it would seem that the vitality of the cholera spirillum in milk depends largely upon the reaction: the more quickly the milk becomes sour the more quickly does the organism become inert; while the longer the milk retains its neutral, or only very slightly acid, reaction, the longer do the cholera organisms that may be present in it retain their power of multiplication.

According to Laser,[1] the cholera organism retains its vitality in butter for about seven days; it is therefore possible for the disease to be contracted by the use of butter that has in any way been in contact with cholera material.

In regard to the antagonism between the cholera spirillum and other organisms with which it may come in contact, the experiments of Kitasato[2] led him to conclude that no organism has been found which, when growing in the same culture-medium with it, possessed the power of depriving it of vitality *within a short time*. On the other hand, the experiments showed that there were quite a number of other organisms the development of which was checked, and in some cases their vitality was completely destroyed, when growing in the same medium with the cholera spirillum.

From this it would appear that the disappearance of the cholera spirillum from mixed cultures and from the evacuations in the short time mentioned, is due more

[1] Zeitschrift für Hygiene. Bd. x. S. 513. [2] Ibid., Bd. vi. S. 1.

to unfavorable nutritive conditions than to the direct action of the other organisms present.

When completely dried, according to Koch, the cholera spirillum does not retain its vitality longer than twenty-four hours, but by others its vitality is said to be destroyed by an absolute drying of three hours. In moist conditions, as in artificial cultures, vitality may be retained for many months; though repeated observations lead us to believe that under these circumstances virulence is diminished. According to Kitasato,[1] they retain their vitality when smeared upon thin glass cover-slips and kept in the moist chamber for from 85 to 100 days, and for as long as 200 days when deposited upon bits of silk thread.

In the course of his studies upon the persistency of pathogenic micro-organisms in the dead body von Esmarch[2] found that when the cadaver of a guinea-pig dead after the introduction of cholera organisms into the stomach was immersed in water until decomposition set in, after eleven days, when decomposition was far advanced, it was impossible to find any living cholera spirilla by the ordinary plate methods.

A similar experiment resulted in their disappearance in five days. In another experiment, in which decomposition was allowed to go on without the animal being immersed in water, none could be detected after the fifth day.

Carl Fränkel[3] has shown that an atmosphere of carbonic acid is directly inhibitory to the development of the cholera spirillum, and Percy Frankland[4] states that in an atmosphere of this gas it dies in about eight days.

[1] Zeitschrift für Hygiene, Bd. v. S. 134.
[2] Ibid., Bd. v. S. 332.
[3] Ibid., Bd. vii. S. 1.
[4] Ibid., Bd. vi. S. 13.

In an atmosphere of carbon monoxide its vitality is lost in nine days, and in general the same may be said for it when exposed to an atmosphere of nitrous oxide gas.

From what has been said, we see that the spirillum of Asiatic cholera, while possessing the power of producing in human beings one of the most rapidly fatal diseases with which we are acquainted, is still one of the least resistant of the pathogenic organisms known to us. Under conditions most favorable to its growth its development is self-limited; it is markedly susceptible to acids, alkalies, other chemical disinfectants, and heat; but when partly dried upon clothing, food, or other objects, it may retain its vitality for a relatively long period of time, and it is more than probable that in this way the disease is often disseminated from points in which it is epidemic or endemic into localities that are free from it.

THE DIAGNOSIS OF ASIATIC CHOLERA BY BACTERIOLOGICAL METHODS.

Because of the manifold channels that are open for the dissemination of this disease it is of the utmost importance that it should be recognized as quickly as possible, for with every moment of delay in its recognition opportunities for its spread multiply. It is essential, therefore, when employing bacteriological means for making the diagnosis, to bear in mind those biological and morphological features of the organism that appear most quickly under artificial methods of cultivation, and which, at the same time, may be considered as characteristic of it, viz., its peculiar morphology and grouping; the much greater rapidity of its growth over that of other bacteria with which it may

be associated; the characteristic appearance of its colonies on gelatin plates and of its growth in stab-cultures in gelatin; its property of producing indol and coincidently nitrites in from six to eight hours in peptone solution at 37° to 38° C.; and its power of causing the death of guinea-pigs in from sixteen to twenty-four hours when introduced into the peritoneal cavity, death being preceded by symptoms of extreme toxæmia, characterized by prostration and gradual and continuous fall in the temperature of the animal's body.

In 1893 Koch[1] called attention to a plan of procedure that comprehends the points just enumerated. By its employment the diagnosis can be established in the majority of cases of Asiatic cholera in from eighteen to twenty-two hours. In general, the steps to be taken and points to be borne in mind are as follows: the evacuations should be examined *as soon as possible* after they have been passed.

Microscopic Examination.—1. From one of the small slimy particles seen in the semi-fluid evacuations prepare a cover-slip preparation in the ordinary way and stain it. If, upon microscopic examination, *only* curved rods, or curved rods greatly in excess of all other forms, are present, the diagnosis of Asiatic cholera is *more than likely* correct; and particularly is this true if these organisms are arranged in irregular linear groups with the long axes of all the rods pointing in nearly the same direction—that is to say, somewhat as minnows arrange themselves when swimming up stream in schools. (Koch.)

In 1886 Weisser and Frank[2] expressed their opinion

[1] Zeitschrift für Hygiene und Infiktionskleiten, 1893, Bd. xiv. S. 319.
[2] Ibid., 1886, Bd. i. S. 379.

upon the value of microscopic examination in these cases as follows:

a. In the majority of cases microscopic examination is sufficient for the detection of the presence of the comma bacillus in the intestinal evacuations of cholera patients.

b. Even in the most acute cases, running a very rapid course, the comma bacillus can always be found in the evacuations.

c. In general, the number of cholera spirilla present is greater the earlier death occurs; when death is delayed, and the disease continues for a long period, their number is diminished.

d. Should the patient not die of cholera, but of some other disease, such as typhoid fever, that may be engrafted upon it, comma bacilli may disappear entirely from the intestines.

2. From another slimy flake prepare a set of gelatin plates. Expose them to a temperature of from 20° to 22° C., and after sixteen, twenty-two, and thirty-six hours observe the appearance of the colonies. Usually after about twenty-two hours the colonies of this organism can easily be identified by one familiar with them.

3. With another slimy flake start a culture in a tube of peptone solution—either the solution of Dunham or, as Koch proposes, a solution of double the strength of that of Dunham (Witte's peptone is to be used, as it gives the best and most constant results). Keep this at from 37° to 38° C., and at the end of from six to eight hours prepare cover-slips from the *upper layers* (without shaking) and examine them microscopically. If comma bacilli were present in the original material, and are capable of multiplication, they will be found in this local-

ity in almost pure culture. After the microscopic examination prepare a second peptone culture from the *upper layers* of the one just examined, also a set of gelatin plates, and with what remains make the test for indol by the addition of 10 drops of concentrated sulphuric acid for each 10 c.c. of fluid contained in the tube. If comma bacilli are growing in the tube, the rose color characteristic of the presence of indol should appear.

By following this plan "a bacteriologist who is familiar with the morphological and biological peculiarities of this organism should make a more than *probable* diagnosis at once by microscopic examination alone, and a *positive* diagnosis in from twenty to, at most, twenty-four hours after beginning the examination." (Koch.)

In certain doubtful cases the organisms are present in the intestinal canal in very small numbers, and microscopic examination is not, therefore, of so much assistance. In these cases plates of agar-agar, of gelatin, and cultures in the peptone solution should be made.

The plates of agar-agar should not be prepared in the usual way, but the agar-agar should be poured into Petri dishes and allowed to solidify, after which one of the slimy particles may be smeared over its surface. The comma bacillus, being markedly aërobic, develops very much more readily when its colonies are located upon the surface then when in the depths of the medium. A point to which Koch calls attention, in connection with this step in manipulation, is the necessity of having the surface of the agar-agar free from the water squeezed from it when it solidifies, as the presence of the water interferes with the development of the colonies at isolated points and causes them to

become confluent. To obviate this he recommends that the agar-agar be poured into the plates and the water allowed to separate from the surface at the temperature of the incubator before they are used. It is wise, therefore, when one is liable to be called on for such work as this, to keep a number of sterilized plates of agar-agar in the incubator ready for use, just as sterilized *tubes* of the media are always ready at hand. The advantage of using the agar plates is the higher temperature at which they can be kept, and consequently a more favorable condition for the development of the colonies.

As soon as isolated colonies appear they should be examined microscopically for the presence of bacteria having the morphology of the one we are seeking, and as soon as such is detected gelatin plates and cultures in peptone solution (for the indol reaction) should be made. The peptone culture from the original material should be examined microscopically from hour to hour after the sixth hour that it has been in the incubator. The material taken for examination should always come from *near the surface* of the fluid, and care should be taken not to shake the tube. As soon as comma bacilli are detected in considerable numbers in the upper layers of the fluid agar-agar plates and fresh peptone cultures should be made from them. In from ten to twelve hours at 37° C. the colonies will develop on the agar-agar plates to a size sufficient for recognition by microscopic examination, and from this examination an opinion can usually be formed. This opinion should always be controlled by cultures in the peptone solution made from each of several single colonies, and finally

the test for the presence or absence of indol in these cultures should be made.

In all doubtful cases, in which only a few curved bacilli are present, or in which irregularities in either the rate or mode of their development occur, pure cultures should be obtained as soon as possible by the agar-agar plate method and by the method of cultivation in peptone solution, and their virulence tested upon animals. For this purpose cultures upon agar-agar from single colonies must be made. From the surface of one of such cultures a large wire-loopful should be scraped and broken up in about one cubic centimetre of bouillon, and the suspension thus made injected by means of a hypodermic syringe directly into the peritoneal cavity of a guinea-pig of about 350 to 400 grammes weight. For larger animals more material is used. If the material injected is from a *fresh* culture of the cholera organism, toxic symptoms at once appear; these have their most pronounced expression in depression of temperature, and if one follows this decline in temperature from time to time with the thermometer it will be seen to be gradual and continuous from the time of injection to the death of the animal (Pfeiffer[1]), which occurs in from eighteen to twenty-four hours after the operation.

In general, this is the procedure employed in the Institute for Infectious Diseases at Berlin, under Koch's direction.

[1] Loc. cit.

CHAPTER XXIII.

Spirilla of interest, historically and otherwise, that have been confounded with the spirillum of Asiatic cholera—Their peculiarities and differential features—*Vibrio proteus*, or bacillus of Finkler and Prior—*Spirillum tyrogenum*, or cheese spirillum of Deneke—The spirillum of Miller—*Vibrio Metchnikovi*.

VIBRIO PROTEUS (FINKLER-PRIOR BACILLUS).

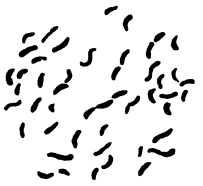
FINKLER AND PRIOR were the first to question experimentally the significance of the presence of Koch's comma bacillus in Asiatic cholera, claiming to have found it in the dejections of individuals suffering from other maladies, particularly *cholera nostras*. The morphological and biological differences between the organism that Finkler and Prior discovered and the comma bacillus described by Koch, are, however, so pronounced as to warrant the opinion that the confusion arose through imperfect and untrustworthy methods of experimentation. Subsequently Finkler and Prior retracted their claims of identity for the two organisms, and held that the bacterium with which they were dealing was peculiar to *cholera nostras*—an opinion which, in the light of later work, was also proved to be without foundation.

The characteristics of the spirillum of Finkler and Prior are as follows:

MORPHOLOGY.—It is thicker and longer than the spirillum of Asiatic cholera; it is often thicker at the middle than at the poles; it forms, like the "comma bacillus," screw-like, twisted threads. (Fig. 77.)

It is supplied with a single flagellum at one of its ends, and is therefore motile.

FIG. 77.



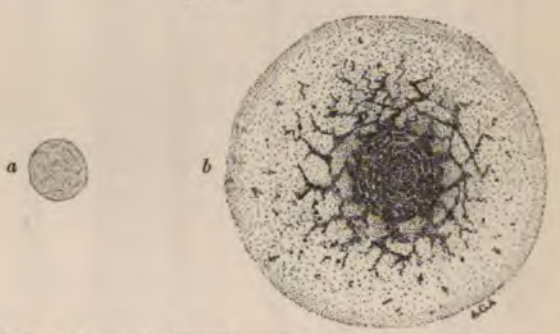
Vibrio proteus (Finkler-Prior bacillus), from culture on agar-agar twenty-four hours old.

It, like the comma bacillus, readily undergoes degenerative changes under conditions unfavorable to growth, and presents the variety of shapes grouped under the head "involution-forms." According to Buchner, this is especially the case when the medium in which they are growing contains glucose (5 per cent.) or glycerin (2 per cent.).

CULTURAL PECULIARITIES.—On *gelatin plates* the development of its colonies is far more rapid, and liquefaction far more extensive, than in the case of the cholera spirillum. After twenty-two to twenty-four hours in this medium at 20° to 22° C. the average size of the colonies is about double that of the comma bacillus. The colonies are darker and denser, and do not present under a low-power lens the same degree of granulation and subsequent lobulation, and they do not become serrated or scalloped around the margin, as is the case with Koch's organism. After twenty-two to twenty-four hours they are usually nearly round, regularly granular, and more or less sharply defined. (See Fig. 78, *a*.) At times they may show indefinite markings or creases, somewhat suggestive of lobulations. After

forty-eight hours on gelatin they usually range from one to three millimetres (some even larger) in diameter, and appear as sharply cut, saucer-shaped pits of liquefaction, in the most dependent portion of which lies a dense, irregular mass, the colony proper. Under a low magnifying power they present at this stage an appearance similar to that shown in Fig. 78, *b*, the central dense mass representing the colony, and the irregular,

FIG. 78.

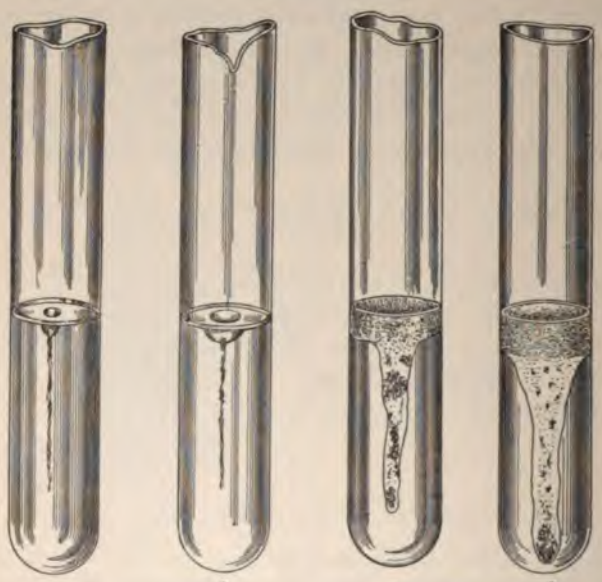


Colonies of the Finkler-Prior bacillus on gelatin. × about 75 diameters. *a*. After twenty-two hours at 20° to 22° C. *b*. After forty-eight hours at 20° to 22° C.

ragged lines surrounding it being shreds that have become torn away as it sank into the liquid caused by its growth. The zone surrounding it, extending to the periphery, is somewhat cloudy, and is simply liquefied gelatin. There is a marked tendency for the liquefaction to spread laterally and for the colonies to run together, so that, even on plates containing few colonies, in sixty to seventy-two hours at from 20° to 22° C., the entire gelatin is usually converted into a yellowish-white fluid. Under these conditions growth is accompanied by a marked aromatic odor, impossible to

describe; this is especially the case when liquefaction is far advanced.

FIG. 79.



Stab-culture of the Finkler-Prior bacillus in gelatin, at 18° to 20° C. *a*. After twenty-four hours. *b*. After forty-eight hours. *c*. After seventy-two hours. *d*. After ninety-six hours.

In *stab-cultures* in gelatin at the room-temperature, liquefaction is noticed about the upper part of the needle-track in twenty-four hours; this gradually increases, until at the expiration of two or three days the entire upper portion of the gelatin has become converted into a cloudy fluid, whereas at the lower part of the canal the liquefaction progresses less rapidly, but is still much more marked than that seen as a result of the growth of Koch's spirillum. Indeed,

under these circumstances there is no similarity whatever between the growth of the two organisms. (See *a*, *b*, *c*, *d*, Fig. 79, and compare them with corresponding cuts in Fig. 76.)

Ragged, more or less dense masses, fragments of the colony proper, are usually seen scattered through the cloudy liquefied gelatin.

On nutrient agar-agar there is nothing particularly characteristic about its growth, appearing only as a moist, grayish or yellowish-gray deposit.

On potato a pale, yellowish-gray deposit appears; after forty-eight to seventy-two hours this becomes moist, glazed, and marked by lobulations, and surrounded by an irregular, colorless zone of growth that is much less moist than that forming the central area. It grows well on potato at ordinary room-temperature.

It causes liquefaction of solidified blood-serum and of coagulated egg-albumin.

In milk to which neutral litmus tincture has been added the blue color assumes a pink tinge in from two to three days at 37° to 38° C.

It does not form indol.

It does not cause fermentation of glucose.

In peptone solution containing rosolic acid the color is somewhat deepened after four or five days at 37° C.

Experiments upon Animals.—The ordinary methods of inoculation show this organism to be without pathogenic properties. Injections, subcutaneous and intravascular and directly into the stomach, give negative results. When introduced into the stomach of guinea-pigs by the method employed by Koch in his cholera experiments, Finkler and Prior had 3 out of

10 animals, and Koch 5 out of 15 animals so treated to die.

In 1885, 1886, and 1887 Franck[1] examined seven cases that clinically presented the condition of *cholera nostras;* in none of these seven cases was the organism of Finkler and Prior, which they claimed to be the cause of the disease, found. In all cases the results of bacteriological examination, in so far as the constant presence of an organism that might stand in causal relation to the disease was concerned, were negative. Only the ordinary intestinal bacteria were detected.

SPIRILLUM TYROGENUM (CHEESE SPIRILLUM OF DENEKE).

Another spiral form, likewise forming short, comma-shaped segments in the course of its growth (Fig. 80), is that found by Deneke in old cheese. It is a little smaller than Koch's spirillum. It is motile and has but a single flagellum, attached to one of its ends. It liquefies gelatin more rapidly than does Koch's organism. It possesses no characteristic grouping, as can be seen in impression cover-slips of its colonies. It does not form spores. On gelatin plates its colonies develop very rapidly as saucer-shaped depressions; after twenty-four hours they vary from 1 to 4 mm. in transverse diameter. To the naked eye they are almost transparent, and are usually marked by a denser centre and peripheral zone, the space between being quite clear. They are not regularly round in all cases. A peculiar aromatic odor accompanies their growth on gelatin. Under low magnifying power the smallest colonies are irregularly round in outline, their borders being often

[1] Zeitschrift für Hygiene, 1888, Band iv. S. 207.

rough and broken, and the body of the colony is frequently marked by creases or ridges that give it a lobulated appearance. The larger colonies under the

FIG. 80.

Deneke's cheese spirillum, *spirillum tyrogenum*. From agar-agar culture twenty-four hours old.

same lens appear as granular patches, a little denser at the periphery and centre than at the intermediate portions. The periphery gradually fades away and no

FIG. 81.

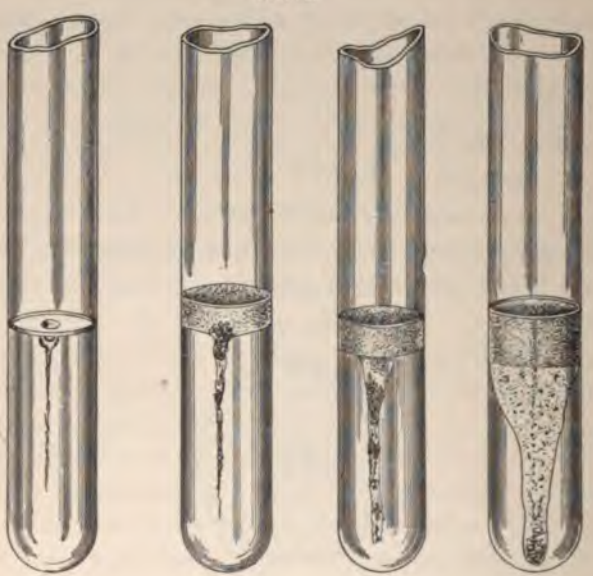
Colony of *spirillum tyrogenum* on gelatin, twenty-four hours old.

distinct circumference can be made out. (See Fig. 81.) The colonies of an intermediate size, about which liquefaction is just beginning to be apparent, show a dense granular centre, the colony itself, and round about it a delicate, granular developmental zone.

In stab-cultures in gelatin liquefaction is rapid, causing at the end of twenty-four hours a cup-shaped depression at the top of the needle-track, the superficial area

of which is about half that of the gelatin in the tube. (Fig. 82, *a*.) The liquefying process spreads laterally, and at the end of forty-eight hours the whole upper portion of the gelatin may have become liquid. (Fig. 82, *b*.) This process continues along the track of the

FIG. 82.



Stab-culture of Deneke's cheese spirillum in gelatin, at 18° to 20° C.
a. After twenty-four hours. *b*. After forty-eight hours. *c*. After seventy-two hours. *d*. After ninety-six hours.

needle, and after seventy-two and ninety-six hours the appearances shown in Fig. 82, *c* and *d*, will be produced.

There is nothing particularly characteristic about its growth upon agar-agar.

On potato a moist, glazed, yellowish, and, at points,

brownish-yellow growth appears that is surrounded by a drier, colorless zone. It is not lobulated.

In milk containing neutral litmus tincture a pink color appears after two to three days at 37° C.; after four days the milk is almost decolorized and coagulation of the casein is beginning, with a layer of clear whey above it. During the subsequent twenty-four hours complete separation of the contents of the tube into clot and whey takes place.

In Dunham's peptone solution it does not form indol, and the reaction for this body does not appear with either sulphuric acid alone or plus sodium nitrite.

It causes liquefaction of both coagulated blood-serum and egg-albumin.

There is no pellicle formed as a result of its growth in bouillon.

It does not produce fermentation of glucose.

In rosolic-acid-peptone solution its growth causes intensification of the red color in four or five days at 37° C.

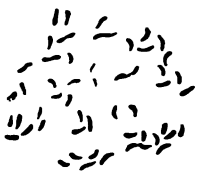
By Koch's method of introducing cultures into the stomach of a guinea-pig this organism produced the death of three out of fifteen animals experimented upon —the deaths being due most probably to the toxic action of the products of growth that were introduced with the organisms rather than to any pathogenic powers possessed by the organism itself.

MILLER'S SPIRILLUM.

Another spirillum that has been likened to that of Koch is the one obtained by Miller from a carious tooth. It has so many characteristics in common with the organism of Finkler and Prior that Miller was inclined

to consider them identical. In morphology they are indistinguishable. (See Fig. 83.) It grows rapidly,

FIG. 83.



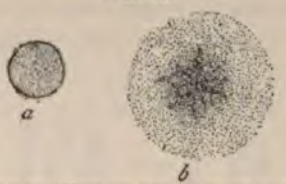
Spirillum of Miller. From agar-agar culture twenty-four hours old.

and, like the spirillum of Finkler and Prior, causes rapid liquefaction of gelatin, with the coincident production of a peculiar aromatic odor.

The colonies on gelatin plates appear after twenty-four hours as small, transparent pits of liquefaction, in the centre of which can be seen a minute white point, the colony itself. Under a low lens the largest of these points are uniformly granular and regularly round, and, as a rule, are surrounded by a peripheral zone a little darker than the central portion of the colony. The circumference is delicately fringed with short, cilia-like prolongations of growth which are not, as a rule, straight, but are twisted in all directions and can only be detected upon very careful examination. (See *a*, Fig. 84.) When located deep in the gelatin the colonies are round, sharply circumscribed, of a pale yellowish or greenish-yellow color, and marked by very delicate irregular lines or ridges. After forty-eight hours the plate containing many colonies is entirely liquefied, while that containing only a few shows the presence of round, sharply-cut, shallow pits of liquefaction that measure from 2 to 10 mm. in diameter. They are a little denser

at the centre than at the periphery, and the dense centre is not sharply circumscribed, but fades off into what has the appearance of a delicate film. (See *b*, Fig. 84.) As

FIG. 84.



Colonies of Miller's spirillum on gelatin, at 20° to 22° C. × about 57 diameters.

a. Colony just beneath the surface of the gelatin. *b*. Colony on the surface of the gelatin.

the colonies become older they are sometimes marked by irregular radii extending from periphery to centre, like the spokes of a wheel.

In stab-cultures in gelatin it rapidly produces liquefaction, both at the surface and along the needle-track, and in most respects gives rise to a condition very like that resulting from the growth of Finkler and Prior's spirillum, though differing from it in certain details. (See *a*, *b*, *c*, *d*, Fig. 85.)

On agar-agar nothing of special interest appears as a result of its development.

On potato its growth is very like that of the cholera spirillum, viz., it appears at 37° C. as a dry, white patch that lies quite flat upon the surface and can often only be seen when the tube is held to the light at a particular angle.

Its growth in bouillon is not characteristic. It does not form a pellicle.

It causes liquefaction of both coagulated blood-serum and egg-albumin.

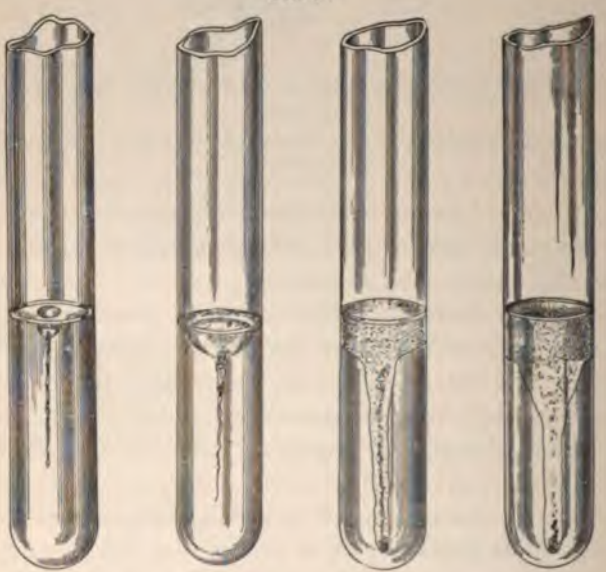
It does not produce indol.

It does not cause fermentation of glucose.

It is non-motile.

In milk containing blue litmus tincture it causes almost complete decolorization in from three to four days at 37° C., with coincident coagulation of the

FIG. 85.



Stab-culture of Miller's spirillum in gelatin, at 18° to 20° C.

a. After twenty-four hours. *b*. After forty-eight hours. *c*. After seventy-two hours. *d*. After ninety-six hours.

casein and the formation of a layer of clear whey about it.

It causes the red color of rosolic-acid-peptone solution to become somewhat intensified after four or five hours at 37° C.

Of twenty-one animals treated with this organism by Koch's method of inoculation only four died.

VIBRIO METCHNIKOVI.

The spirillum that simulates very closely the comma bacillus of cholera in its morphological and cultural peculiarities, but which is still easily distinguished from it, is that described by Gamaleia[1] under the name of

FIG. 86.

Vibrio Metchnikovi from agar-agar culture, twenty-four hours old.

vibrio Metchnikovi. It was found *post mortem* in a number of fowls that had died in the poultry-market of Odessa, and the experiments of the discoverer led him to believe that it was related etiologically to the gastro-enteritis from which the chickens had been suffering.

Morphologically it appears as short, curved rods and as longer, spiral-like filaments. It is usually thicker than Koch's spirillum and is at times *much* longer, while again it is seen to be shorter. It is usually more distinctly curved than the "comma bacillus." (Fig. 86.)

It is supplied with a single flagellum at one of its extremities, and is therefore motile.

It does not form spores.

It is aërobic.

Its growth upon gelatin plates is usually character-

[1] Annales de l'Institut Pasteur, 1888, tome ii., pp. 482, 552.

ized, according to Pfeiffer, by the appearance of two kinds of liquefying colonies, one strikingly like those of the Finkler-Prior organism, the other very similar to those produced by Koch's comma bacillus, though in both cases the liquefaction resulting from the growth of this organism is more energetic than that common to the spirillum of Asiatic cholera. After from twenty-four to thirty hours the medium-size colonies, when examined under a low power of the microscope, show a yellowish-brown, ragged central mass surrounded by a zone of liquefaction that is marked by a border of delicate radii. (Fig. 87.)

FIG. 87.

Colony of *vibrio Metchnikovi* in gelatin, after thirty hours at 20° to 22° C. × about 75 diameters.

In gelatin stab-cultures the growth has much the same general appearance as that of the cholera spirillum, but is exaggerated in degree. The liquefaction is far more rapid, and the characteristic appearance of the growth is lost in from three to four days. (See *a*, *b*, *c*, *d*, Fig. 88.) Development and liquefaction along the deeper parts of the needle-track are much more pronounced than is the case with the "comma bacillus."

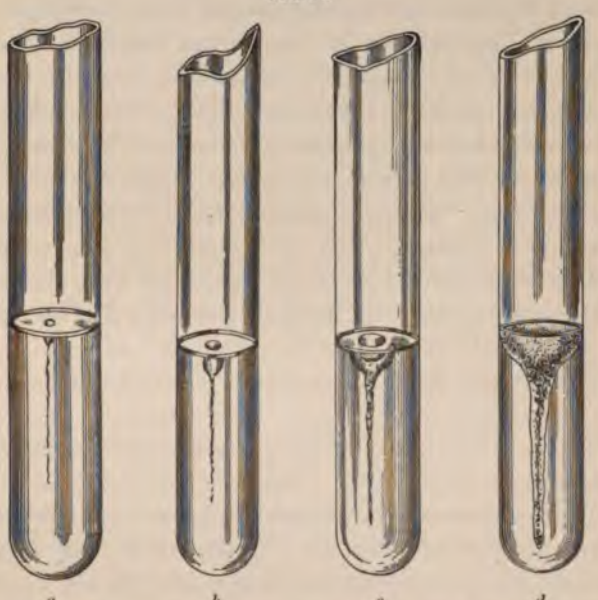
Its growth on agar-agar is rapid; after twenty-four to forty-eight hours a grayish deposit appears, which has a tendency to become yellowish with age.

· potato at 37° C. its growth is seen as a moist, ·lored patch, surrounded by a much paler zone.

The whole growth is so smooth and glistening that it has somewhat the appearance of being varnished.

In bouillon it quickly causes opacity, with the ultimate production of a delicate pellicle upon the surface.

FIG. 88.



Stab-culture of *vibrio Metchnikovi* in gelatin, at 18° to 20° C.

a. After twenty-four hours. *b*. After forty-eight hours. *c*. After seventy-two hours. *d*. After ninety-six hours.

It causes liquefaction of blood-serum, the liquefied area being covered by a dense, wrinkled pellicle.

When grown in peptone solution it produces indol and coincidently nitrites, so that the rose-colored reaction characteristic of indol is obtained by the addi-

tion of sulphuric acid alone. The production of indol by this organism is usually *greater* than that common to the comma bacillus under the same circumstances.

In milk it causes an acid reaction with coagulation of the casein. The coagulated casein collects at the bottom of the tube in irregular masses, above which is a layer of clear whey. If blue litmus has been added to the milk, the color is changed to pink in from twenty-four to thirty hours, and after forty-eight hours decolorization and coagulation occur. The clots of casein are not re-dissolved. After about a week the acidity of the milk is at its maximum, and the organisms quickly die.

It causes the red color of the rosolic-acid-peptone solution to become very much deeper after four or five days at 37° C.

It does not cause fermentation of glucose with production of gas.

It is killed in five minutes by a temperature of 50° C. (Sternberg.)

It is pathogenic for chickens, pigeons, and guinea-pigs. Rabbits and mice are affected only by very large doses.

Gamaleia states that chickens affected with the choleraic gastro-enteritis of which this organism is the cause, are usually seen sitting quietly with ruffled feathers. They suffer from diarrhœa, but there is no elevation of temperature. Hyperæmia of the entire gastro-intestinal tract is seen at autopsy. The other internal organs do not, as a rule, present anything abnormal to the naked eye. The intestinal canal contains yellowish fluid with which blood may be mixed. In adult chickens

the spirilla are not found in the blood, but in young ones they are usually present in small numbers.

After the introduction into the pectoral muscle of a very small quantity of a culture of this organism pigeons succumb in from eight to twenty hours. The most conspicuous *post-mortem* lesion is found at the site of inoculation. The muscle is marked by yellow, necrotic stripes; is more or less œdematous; is swollen, and contains the vibrios in enormous numbers. The intestines are usually filled with fluid contents, which may or may not be blood-stained; the walls of the intestines are often injected with blood, and occasionally markedly so. The conditions of the other internal viscera are inconstant. In fatal cases the vibrios are present in large numbers in the blood and internal organs. In pigeons that survive inoculation the organisms may be found only at the site of inoculation, or very sparingly in the blood also. These animals usually exhibit immunity from subsequent inoculations. In certain instances the results of infection are chronic; the inoculated pectoral muscle atrophies, the pigeon loses in weight and finally dies after one or two weeks. In these cases the organisms are usually absent from the blood and internal organs, and may even be absent from the site of inoculation, or, if present, in only very small number.

Guinea-pigs usually die in from twenty to twenty-four hours after subcutaneous inoculation. At autopsy an extensive œdema of the subcutaneous tissues about the seat of inoculation is seen, and there is usually a necrotic condition of the tissues in the vicinity of the point of puncture. As the blood and internal organs of both pigeons and guinea-pigs contain the vibrios in

large numbers, the infection in these animals takes, therefore, the form of acute, general septicæmia.

The blood-serum of both pigeons and guinea-pigs that have survived inoculation with this organism—*i. e.*, that have acquired immunity from it—are bactericidal *in vitrio* for this organism. It also possesses a certain degree of immunity-conferring property, as may be demonstrated by injecting it into normal pigeons and guinea-pigs that are subsequently to be inoculated with virulent cultures.

Very old cultures of this organism in bouillon become distinctly alkaline in reaction. At this stage they contain a toxin that is markedly active for susceptible animals. This toxin is not dissolved in the fluid to any extent, but is apparently in intimate association with the proteid matters composing the bacteria.

Gastro-enteritis may be produced in both chickens and guinea-pigs by feeding them with food with which cultures of this organism have been mixed. (Gamaleia.)

In the autumn of 1896 the author isolated from the water of the Schuylkill River at Philadelphia a spirillum that is pathogenic for pigeons and guinea-pigs, and that possesses so many of the other characteristics common to the group of spirilla of which the cholera spirillum is the most important member, as to justify the opinion that they are of the same family. That it is not identical with the cholera spirillum is evident, for the reason that the latter produces cholera, while the *vibrio Schuylkilliensis* manifestly does not.[1]

[1] For the detailed description of this organism, see Journal of Experimental Medicine, vol. i. p. 419; also Transactions of the Association of American Physicians, 1896, vol. xi. p. 394.

NOTE.—Since the epidemic of cholera in Hamburg quite a number of curved or spiral organisms, somewhat like the cholera spirillum, have been discovered. For the descriptions of these the reader is referred to current bacteriological literature.

CHAPTER XXIV.

Study of *bacillus anthracis*, and of the effects produced by its inoculation into animals—Peculiarities of the organism under varying conditions of surroundings.

THE discovery that the blood of animals suffering from splenic fever, or anthrax, always contained minute rod-shaped bodies (Pollender, 1855; Davaine, 1863), led to a closer study of this disease, and has resulted probably in contributing more to our knowledge of bacteriology in general than work upon any of the other infectious maladies.

The outcome of these investigations is that a rod-shaped micro-organism, now known as *bacillus anthracis*, is always present in the blood of animals suffering from this disease; that this organism can be obtained from the tissues of these animals in pure cultures; and that these artificial cultures of *bacillus anthracis* when introduced into the bodies of susceptible animals can again produce a condition identical with that found in the animal from which they were obtained. The disease is a true septicæmia, and after death the capillaries throughout the body are always found to contain the typical rod-shaped organism in larger or smaller numbers.

This organism, when isolated in pure culture, is a bacillus which varies considerably in length, ranging from short rods 2 to 3 μ in length to longer threads 20 to 25 μ in length. In breadth it is from 1 to

1.25 μ. Frequently very long threads, made up of several rods joined end to end, are seen.

When obtained directly from the body of an animal it is usually in the form of short rods *square at the ends.* If highly magnified, the ends are seen to be a trifle thicker than the body of the cell and somewhat indented or concave, peculiarities that help to distinguish it from certain other organisms that are somewhat like it morphologically. (See Fig. 89.)

FIG. 89.

Bacillus anthracis, highly magnified to show swellings and concavities at extremities of the single cells.

When cultivated artificially at the temperature of the body the bacillus of anthrax presents a series of very interesting stages.

The short rods develop into long threads, which may be seen twisted or plaited together like ropes, each thread being marked by the points of juncture of the short rods composing it. (Fig. 90, *a* and *b*.) In this condition it remains until alterations in its surroundings, the most conspicuous being diminution of its nutritive supply, favor the production of spores. When this stage begins changes in the protoplasm of the bacilli may be noticed; they become marked by irregular, granular bodies, which eventually coalesce into glistening, oval spores, one of which lies in nearly every segment of the long thread, and gives to the thread the

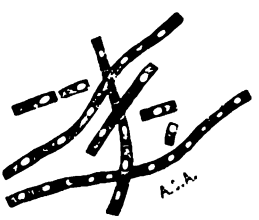
appearance of a string of glistening beads. (Fig. 91.) In this stage they remain but a short time. The chains of spores, which are held together by the remains of

FIG. 90.

Bacillus anthracis. Plaited and twisted threads seen in fresh growing cultures. × about 400 diameters.

the cells in which they formed, become broken up, and eventually nothing but free oval spores, and here and there the remains of mature bacilli which have undergone degenerative changes, can be found. In this condition the spores, capable of resisting deleterious influ-

FIG. 91.



Threads of *bacillus anthracis* containing spores. × about 1200 diameters.

ences, remain and, unless their surroundings are altered, continue in this living, though inactive, condition for a very long time. If again placed under favorable conditions, each spore will germinate into a mature cell,

and the same series of changes will be repeated until the surroundings become again gradually unfavorable to development, when spore-formation again takes place. Spore-formation occurs only at temperatures ranging from 18° to 43° C., 37.5° C. being the optimum. Under 12° C. they are not formed. This organism does not form spores in the tissues of the living animal, its usual condition at this time being that of short rods; occasionally, however, somewhat longer forms may be seen.

The bacillus of anthrax is not motile.

GROWTH ON AGAR-AGAR.—Colonies of this organism, as seen upon agar-agar, present a very typical appearance, from which they have been likened unto the head of Medusa. From a central point which is

FIG. 92.

Colony of *bacillus anthracis* on agar-agar.

more or less dense, consisting of a felt-like mass of long threads irregularly matted together, the growth continues outward upon the surface of the agar-agar. (Fig. 92.) It is made up of wavy bundles in which the threads are seen to lie parallel or are twisted in strands like those of a rope; sometimes they have a plaited arrangement. (See Fig. 90.) These bundles twist and cross in all directions, and eventually dis-

appear at the periphery of the colony. At the extreme periphery of the colonies it is sometimes possible to trace single bundles of these threads for long distances across the surface of the agar-agar. The colony itself is not circumscribed in appearance, but is more or less irregularly fringed or ragged, or scalloped. To the naked eye they look very much like minute pellicles of raw cotton that have been pressed into the surface of the agar-agar.

As the colonies continue to grow they become more and more dense and opaque, and granular and rough on the surface. When touched with a sterilized needle one experiences a sensation that suggests somewhat their matted structure. They are never moist or creamy. The bit that is taken up with the needle is always more or less ragged.

GELATIN.—The colonies on gelatin at the earliest stages also present the same wavy appearance; but this characteristic soon becomes in part destroyed by the liquefaction of the gelatin which is produced by the growing organisms. This allows them to sink to the bottom of the fluid, where they lie as an irregular mass. Through the fluid portion of the gelatin may be seen small clumps of growing bacilli, which look very much like bits of cotton-wool.

BOUILLON.—In bouillon the growth is characterized by the formation of flaky masses, which also have very much the appearance of bits of raw cotton. Microscopic examination of one of these flakes reveals the twisted and plaited arrangement of the long threads.

POTATO.—On this medium it develops rapidly as a dull, dry, granular, whitish mass, which is more or less limited to the point of inoculation. On potato, at the

temperature of the incubator, spore-formation may be easily observed.

STAB- AND SLANT-CULTURES.—Stab- and slant-cultures on agar-agar present in general the appearances given for the colonies, except that the growth is much more extensive. The growth is always more pronounced on the surface than down the track of the needle.

On gelatin it causes liquefaction, which begins on the surface at the point inoculated and spreads outward and downward.

It grows best with access to oxygen, and very poorly when the supply of that gas is interfered with.

Under favorable conditions of aëration, nutrition, and temperature its growth is rapid.

Under 12° C. and above 45° C. no growth occurs. Its optimum temperature is that of the human body, viz., 37°–38° C.

The spores of the anthrax bacillus are very resistant to heat, though the degree of resistance varies with spores of different origin. Von Esmarch found that anthrax spores from some sources were readily killed by an exposure of one minute to the temperature of steam, whereas spores from other sources resisted this temperature longer, in some cases as long as twelve minutes.

STAINING.—Anthrax bacilli stain readily with the ordinary aniline dyes. In tissues their presence may also be demonstrated by the ordinary aniline staining-fluids or by Gram's method. They may also be stained in tissues with a strong watery solution of dahlia, after which the sections are decolorized in 2 per cent. sodium carbonate solution, washed in water, dehydrated in alcohol, cleared in xylol, and mounted in bal-

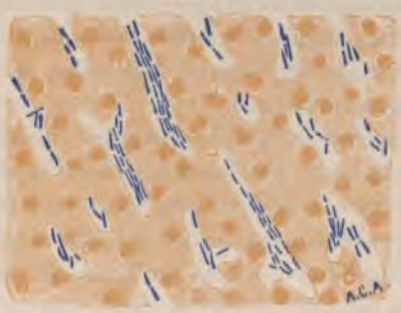
sam. This leaves the bacilli stained, while the tissues containing them are decolorized; or the latter may be stained a contrast-color—with eosin, for example—after dehydration in alcohol and before clearing in xylol. In this case they must be washed again in alcohol before using the xylol. In a preparation treated in this way the rod-shaped organisms are of a purple color, and will be seen in the capillaries of the tissues, while the tissues themselves are of a pale rose color.

Inoculation into Animals.—Introduce into the subcutaneous tissues of the abdominal wall of a guinea-pig or rabbit a portion of a pure culture of *bacillus anthracis*. The animal usually succumbs in from thirty-six to forty-eight hours. Little or no reaction at the immediate point of inoculation will be noticed; but beyond this, extending for a long distance over the abdomen and thorax, the tissues will be markedly œdematous. Here and there, scattered through this œdematous tissue, small ecchymoses will be seen. The underlying muscles are pale in color. Inspection of the internal viscera reveals no very marked macroscopic changes except in the spleen. This is enlarged, dark in color, and soft. The liver may present the appearance of cloudy swelling; the lungs may be red or pale red in color; the heart is usually filled with blood. No other changes can be seen by the naked eye.

Prepare cover-slip preparations from the blood and other viscera. They will all be found to contain short rods in large numbers. Nowhere can spore-formation be detected. Upon microscopic examination of sections of the organs which have been hardened in alcohol the capillaries are seen to be filled with the bacilli; in some places closely packed in large num-

bers, at other points fewer in number. Usually they are present in largest numbers in those tissues having the greatest capillary distribution and at those points at which the circulation is slowest. They are uniformly distributed through the spleen. The glomeruli of the kidneys and the capillaries of the

FIG. 93.



Anthrax bacilli in liver of mouse. × about 450 diameters. Bacilli stained by Gram's method; tissue stained with Bismarck-brown.

lungs are frequently packed with them. The capillaries of the liver contain them in large numbers. (Fig. 93.) Hemorrhages, probably due to rupture of capillaries by the mechanical pressure of the bacilli which are developing within them, not uncommonly occur. When these occur in the mucous membranes of the alimentary tract the blood may escape through the mouth or anus; when in the kidneys, through the uriniferous tubules.

Cultures from the different organs or from the œdematous fluid about the point of inoculation result in growth of *bacillus anthracis.*

The amphibia, dogs, and the majority of birds are not susceptible to this disease. Rats are difficult to

infect. Rabbits, guinea-pigs, white mice, gray house-mice, sheep, and cattle are susceptible. Infection may occur either through the circulation, through the air-passages, through the alimentary tract, or, as we have just seen, through the subcutaneous tissues.

PROTECTIVE INOCULATION.

The most noteworthy application of artificially prepared living vaccines to the protection of animals against infection is seen in connection with anthrax in sheep and in bovines.

By a variety of procedures the virulent anthrax bacillus may be in part or totally robbed of its pathogenic properties. It is through the very mild constitutional disturbance caused in animals vaccinated with such weakened cultures that protection is often afforded against the severer, frequently fatal, form of the infection.

Without reviewing the various methods that have been employed for attenuating the virulence of this organism to a degree suitable for protective vaccination, it will suffice to say that the most satisfactory results have been obtained by long-continued cultivation (ten to thirty days) at a temperature of from 42° to 43° C. In this procedure the spore-free, virulent *bacillus anthracis*, obtained directly from the blood of a recently dead animal, is brought at once into sterile nutrient bouillon in about twenty test-tubes, which are immediately placed in an incubator that is carefully regulated to maintain a temperature of 42.5° C. There should not be a fluctuation of over 0.1° C.

After about a week a tube is removed from the incubator on each successive day and its virulence tested at

once on animals. The degree of attenuation experienced by the cultures grown under these circumstances is determined by tests upon rabbits, guinea-pigs, and mice. The first culture removed may or may not kill rabbits, the most resistant of the three animals used for the test, while it will certainly kill the guinea-pigs and mice; after another two or three days rabbits will no longer succumb to inoculation with the culture last removed from the incubator, while no diminution will as yet be noticed in its pathogenesis for the other two species. After four to seven days more a culture may be encountered that kills only mice, the guinea-pigs escaping; while ultimately, if the experiment be continued, a degree of attenuation may be reached in which the organism has not even the power of killing a mouse, though it still retains its vitality. Investigation of these attenuations shows them to possess all the characteristics of enfeebled anthrax bacilli; they grow slowly and less vigorously when transplanted; they do not form spores when exposed to a high temperature; and microscopically they present evidences of degeneration. When introduced beneath the skin of animals they disseminate but slightly beyond the site of inoculation, and do not, as a rule, cause the general septicæmia that occurs in susceptible animals after inoculation with normal cultures of this organism. In the practical employment of these attenuated cultures for protective purposes two vaccines are employed. These were designated by Pasteur as "first" and "second" vaccines. The "first" is the one that killed only the mice in the preliminary tests; while the "second" is that which killed both mice and guinea-pigs, but failed to kill the rabbit. When larger animals,

such as sheep or cattle, are to be protected by vaccination with these vaccines, a subcutaneous inoculation of about 0.3 c.c. of the first vaccine is usually given. This should be practically without noticeable effect, causing neither rise of body-temperature nor other constitutional or local symptoms. After a period of about two weeks the second vaccine is injected in the same way; this may or may not cause disturbance. In the event of its doing so the symptoms are rarely alarming, and, if the vaccines have been properly prepared and tested before use, they disappear within a short time after the injection.

In the large majority of cases sheep, bovines, horses, and mules may be safely protected against anthrax by the careful practice of this method.

EXPERIMENTS.

Prepare three cultures of anthrax bacilli—one upon gelatin, one upon agar-agar, and one upon potato. Allow the gelatin culture to remain at the ordinary temperature of the room, place the agar-agar culture in the incubator, and the potato culture at a temperature not above 18° to 20° C. Prepare cover-slips from each from day to day. What differences are observed?

Prepare two potato cultures of the anthrax bacillus. Place one in the incubator and maintain the other at a temperature of from 18° to 20° C. Examine them each day. Do they develop in the same way?

From a fresh culture of anthrax bacilli, in which spore-formation is not yet begun (which is the surest

source from which to obtain *non-spore-bearing* anthrax bacilli), prepare a hanging-drop preparation; also a cover-slip preparation in the usual way and stain it with a strong gentian-violet solution; and another cover-slip preparation which is to be drawn through a flame twelve to fifteen times, stained with aniline gentian-violet, washed in iodine solution and then in water. Examine these microscopically. Do they all present the same appearance? To what are the differences due?

Do the anthrax threads, as seen in a fresh, growing, hanging drop, present the same morphological appearance as when dried and stained upon a cover-slip? How do they differ?

Liquefy a tube of agar-agar, and when it is at the temperature of 40° to 43° C. add a very minute quantity of an anthrax culture which is far advanced in the spore-stage. Mix it thoroughly with the liquid agar-agar and from this prepare several hanging drops under strict antiseptic precautions, using the fluid agar-agar for the drops instead of bouillon or salt-solution. Select from among these preparations that one in which the smallest number of spores are present. Under the microscope observe the development of a spore into a mature cell. Describe carefully the developmental stages.

Prepare a 1 : 1000 solution of carbolic acid in bouillon. Inoculate this with virulent anthrax spores. If no development occurs after two or three days at the temperature of the thermostat, prepare a solution of 1 : 1200, and continue until the point is reached at

which the amount of carbolic acid present *just* permits of the development of the spores. When the proper dilution is reached prepare a dozen of such tubes and inoculate one of them with virulent anthrax spores. As soon as development is well advanced transfer a loopful from this tube into a second of the carbolic acid tubes; when this has developed, then from this into a third, etc. After five or six generations have been treated in this way study the spore-production of the organisms in that tube. If it is normal, continue to inoculate from one carbolic acid tube to another, and see if it is possible by this means to influence in any way the production of spores by the organism with which you are working. What is the effect, if any?

Prepare two bouillon cultures, each from *one drop of* blood of an animal dead of anthrax. (Why from the blood of an animal and not from a culture?) Allow one of them to grow for from fourteen to eighteen hours in the incubator; allow the other to grow at the same temperature for three or four days. Remove the first tube after the time mentioned and subject it to a temperature of 80° C. for thirty minutes. At the end of this time prepare four plates from it. Make each plate with one drop from the heated bouillon culture. At the end of three or four days treat the second tube in identically the same way. How do the number of colonies which develop from the two cultures compare? Was there any difference in the time required for their development on the plates?

From a potato culture of anthrax bacilli which has been in the incubator for three or four days scrape

away the growth and carefully break it up in 10 c.c. of sterilized physiological salt-solution. The more thoroughly it is broken up the more accurate will be the results of the experiment. Place this in a bath of boiling water, and at the end of one, three, five, seven, and ten minutes make plates upon agar-agar each with one loopful of the contents of this tube. Are the results on the plates alike?

Determine the exact time necessary to sterilize objects, such as silk or cotton threads, on which anthrax spores have been dried, by the steam method and by the hot-air method.

Prepare a bouillon culture from the blood of an animal just dead of anthrax. After this has been in the incubator for from three to four hours subject it to a temperature of 55° C. for ten minutes. At the end of this time make plates from it and also inoculate a rabbit subcutaneously with it. What are the results? Are the colonies on the plates in every way characteristic?

Inoculate six Erlenmeyer flasks of sterile bouillon, each containing about 35 c.c. of the medium, from either the blood of an animal just dead of anthrax or from a fresh virulent culture in which *no spores* are formed.

Place these flasks in the incubator at a temperature of 42.5° C. At the end of five, ten, fifteen, twenty, twenty-five, or more days remove a flask. Label each flask as it is taken from the incubator with the exact number of days that it has been at the tempera-

ture of 42.5° C. Study each flask carefully, both in its culture-peculiarities and in its pathogenic properties when employed on animals.

Are these cultures identical in all respects with those that have been kept at 37° C.?

If they differ, in what respect is the difference most conspicuous?

Should any of the animals survive the inoculations made from the different cultures in the foregoing experiment, note carefully which one it is, and after ten to twelve days repeat the inoculation, using the same culture; if it again survives, inoculate it with the culture preceding the one just used in the order of removal from the incubator; if it still survives, inoculate it with virulent anthrax. What is the result? How is the result to be explained? Do the cultures which were made from these flasks at the time of their removal from the incubators act in the same way toward animals as the organisms growing in the flasks? Is the action of each of these cultures the same for mice, guinea-pigs, and rabbits?

Prepare a 2 per cent. solution of sulphuric acid in distilled water; suspend in this a number of anthrax spores; at the end of three, six, and nine days at 35° C. inoculate both a guinea-pig and a rabbit. Prepare cultures from this suspension on the third, sixth, and ninth days; when the cultures have developed inoculate a rabbit and a guinea-pig from the culture made on the ninth day. Should the animals survive, inoculate them again after three or four days with a culture made on the sixth day. Do the results appear in any way peculiar?

CHAPTER XXV.

The most important of the organisms found in the soil—The nitrifying bacteria—The bacillus of tetanus—The bacillus of malignant œdema—The bacillus of symptomatic anthrax.

THE NITRIFYING BACTERIA.

By the employment of bacteriological methods in the study of the soil much light has been shed upon the cause and nature of the interesting and momentous biological phenomena there constantly in progress. Of these, the one that is of the greatest importance comprises those changes that accompany the widespread process of disintegration and decomposition, to which reference has already been made. (See Chapter I.) This resolution of dead complex organic compounds into simpler structures that are assimilable as food by growing vegetation is dependent upon the activities of bacteria located in the superficial layers of the ground. It is not a simple process, brought about by a single, specific species of bacteria; but represents a series of metabolic alterations, each definite step of which is most probably the result of the activities of different species or groups of species, acting singly or together (symbiotically). Our knowledge upon the subject is not sufficient to permit us to follow in detail the manifold alterations undergone by dead organic material in the process of decomposition that results in its conversion into inorganic compounds, with the formation of carbonic acid, ammonia, and water as the conspicuous end-products. It suffices

to say that wherever dead organic matters are exposed to the action of the great group of saprophytic bacteria, in which are found many different species, the alterations through which they pass are ultimately characterized by the appearance of these three bodies. When the process of decomposition occurs in the soil, however, it does not cease at this point, but we find still further alterations — alterations concerning more particularly the ammonia. This change in ammonia is characterized by the products of its oxidation, viz., by the formation of nitrous and nitric acids and their salts; it is not a result of the direct action of atmospheric oxygen upon the ammonia, but occurs through the instrumentality of a special group of saprophytes known as the *nitrifying* organisms. They are found in the most superficial layers of the ground, and though more common in some places than in others, they are, nevertheless, present over the entire surface of the earth. The most conspicuous example of the functional activity of this specific form of soil organism is seen in the immense saltpetre-beds of Chili and Peru, where, by the activities of these microscopic plants, nitrates are produced from the ammonia of the fæcal evacuations of sea-fowl in such enormous quantities as to form the source of supply of this article for the commercial world. A more familiar example, though hardly upon such a great scale, is seen in the decomposition and subsequent nitrification of the organic matters of sewage and other impure waters in the process of purification by filtration through the soil, a process in which it is possible to follow, by chemical means, the organic matters from their condition as such through their conspicuous modifications to their ultimate conversion into

ammonia, nitrous and nitric acids. In fact, the same breaking down and building up, resulting ultimately in nitrification, occurs in all nitrogenous matters that are deposited upon the soil and allowed to decay. It is largely through this means that growing vegetation obtains the nitrogen necessary for the nutrition of its tissues, and when viewed from this standpoint we appreciate the importance of this process to all life, animal as well as vegetable, upon the earth.

These very important and interesting nitrifying organisms, of which there appear to be several, have been the subject of much study, and are found to possess peculiarities of sufficient interest to justify a more or less detailed description. For a long time all efforts to isolate them from the soils in which they were believed to be present, and to cultivate them by the processes commonly employed in bacteriological work, resulted in failure; and it was not until it was found that the ordinary methods of bacteriological research were in no way applicable to the study of these bacteria that other, and ultimately successful, methods were devised. By these special devices nitrifying bacteria, capable of oxidizing ammonia to nitric acid, have been isolated and cultivated, and the more important of their biological peculiarities recorded by Winogradsky in Switzerland, by G. C. and P. F. Frankland in England, and by Jordan and Richards in this country. From the similarity of the properties, given by these several observers, of the nitrifying organisms isolated by them, it seems likely that they have all been working with either the same organism or very closely allied species.

The organism generally known as the *nitro-monas* of

Winogradsky is a short, oval, and frequently almost spherical cell. It divides as usual for bacteria, but there is little tendency for the daughter-cells to adhere together or to form chains. In cultures they are commonly massed together, by a gelatinous material, in the form of zoöglœa. They do not form spores, and are probably not motile, though Winogradsky believes he has occasionally detected them in active motion. As has been stated, they do not grow upon ordinary nutrient media, and cannot, therefore, be isolated by the means commonly employed to separate different species of bacteria. The most astonishing property of this organism is its ability to grow and perform its specific fermentative function in solutions devoid of organic matter. It is believed to be able to obtain its necessary carbon from carbonic acid. For its isolation and cultivation Winogradsky recommends the following solution:

Ammonium sulphate	1 gramme.
Potassium phosphate	1 "
Pure water	1000 c.c.

To each flask containing 100 c.c. of this fluid is added from 0.5 to 1 gramme of basic magnesium carbonate suspended in a little distilled water and sterilized by boiling. One of the flasks is then to be inoculated with a minute portion of the soil under investigation, and after four or five days a small portion is to be withdrawn, by means of a *capillary* pipette, from over the surface of the layer of magnesium carbonate and transferred to a second flask, and similarly after four or five days from this to a third flask, and so on. As this medium does not offer conditions favorable to the

growth of bacteria requiring organic matter for their development, those that were originally introduced with the soil quickly disappear, and ultimately only the nitrifying organisms remain. These are seen as an almost transparent film attached to the clumps and granules of magnesium carbonate on the bottom of the flask.

For their cultivation upon a solid medium Winogradsky employs a mineral gelatin, the gelatinizing principle of which is silicic acid. A solution of from 3 to 4 per cent. of silicic acid in distilled water, and having a specific gravity of 1.02, remains fluid and can be preserved in flasks in this condition. (Kühne.) Gelatinization occurs after the addition of certain salts to such a solution, and will be more or less complete according to the proportion of salts added. The salts that have given the best results and the method of mixing them are as follows:

a	Ammonium sulphate	0.4 gramme.
	Magnesium sulphate	0.05 "
	Calcium chloride	trace.
b	Potassium phosphate	0.1 gramme.
	Sodium carbonate	0.6 to 0.9 "
	Distilled water	100 c.c.

The sulphates and chloride (*a*) are mixed in 50 c.c. of the distilled water, and the phosphate and carbonate (*b*) in the remaining 50 c.c., in separate flasks.

Each flask is then sterilized with its contents, which after cooling are mixed; the mixture representing the solution of mineral salts is to be added to the silicic acid, little by little, until the proper degree of consistency is obtained (that of ordinary nutrient gelatin). This part of the process is best conducted in a culture-dish. If it is desired to separate the colonies, as in an ordinary plate, the inoculation and mixing of the mate-

rial introduced must be done before gelatinization is complete; if the material is to be distributed over only the surface of the medium, then the mixture must first be allowed to solidify.

By the use of the silicate-gelatin Winogradsky has isolated from the gelatinous film in the bottom of fluids undergoing nitrification a bacillus which he believes to be associated with the nitro-monas in the nitrifying process.

Our knowledge of these organisms is as yet too incomplete to permit of a complete description. What has been said will serve to indicate the direction in which further studies of the subject should be prosecuted. (For further details, the reader is referred to the original contributions and to current literature on the subject.[1])

In addition to the bacteria concerned in decomposition and nitrification there are occasionally present in the soil micro-organisms possessing disease-producing properties. Conspicuous among these may be mentioned the bacillus of malignant œdema (*vibrion septique* of the French), the bacillus of tetanus, and the bacillus of symptomatic anthrax (*Rauschbrand* (Ger.); *charbon symptomatique* (Fr.)). It is sometimes due to the presence of one or the other of these organisms that wounds to which soil has had access (crushed wounds from the wheels of cars or wagons, wounds received in agricultural work, etc.) are followed by such grave constitutional disturbances.

[1] Winogradsky: Ann. de l'Inst. Pasteur, 1890, tome iv.; 1891, tome v.

Jordan and Richards: Report of State Board of Health of Massachusetts, "Purification of Sewage and Water," 1890, vol. ii. p. 864.

Frankland, G. C. and P. F.: Proceedings of Royal Society, London, 1890, xlvii.

THE BACILLUS OF TETANUS.

In 1884 Nicolaier produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden-earth, and demonstrated that the pus produced at the point of inoculation was capable of reproducing the disease in other mice and rabbits. He did not succeed in isolating the organism in pure culture. In 1884 Carle and Rattone, and in 1886 Rosenbach, demonstrated the infectious nature of tetanus as it occurs in man by producing the disease in animals by inoculating them with secretions from the wounds of individuals affected with the disease. In 1889 Kitasato obtained the bacillus of tetanus in pure culture, and described his method of obtaining it and its biological peculiarities as follows:

METHOD OF OBTAINING IT.—Inoculate several mice subcutaneously with secretions from the wound of a case of typical tetanus. This material usually contains not only tetanus bacilli, but other organisms as well, so that at autopsy, if tetanus results, there may be more or less suppuration at the seat of inoculation in the mice. In order to separate the tetanus bacillus from the others that are present the pus is smeared upon the surface of several slanted blood-serum or agar-agar tubes and placed at 37° to 38° C. After twenty-four hours all the organisms will have developed, and microscopic examination will usually reveal the presence of a few tetanus bacilli, recognizable by their shape, viz., that of a small pin, with a spore representing the head. After forty-eight hours at 38° C. the culture is subjected to a temperature of 80° C. in a water-bath for from three-quarters to one hour. At the end of this time series of

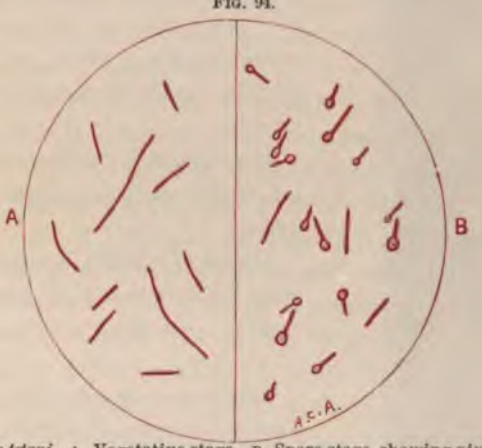
plates or Esmarch tubes of slightly alkaline gelatin are made with very small amounts of the culture and kept in an atmosphere of hydrogen (see page 210). They are then kept at from 18° to 20° C., and at the end of about a week the tetanus bacillus begins to appear in the form of colonies. After about ten days the colonies should not only be examined microscopically, but each colony that has developed in the hydrogen atmosphere should be obtained in pure culture and again grown under the same conditions. The colonies that grow only without oxygen, and which are composed of the pin-shaped organisms, must be tested upon mice. If they represent growths of the tetanus bacillus, the typical clinical manifestations of the disease will be produced in these animals.

In obtaining the organism from the soil much difficulty is experienced. Here are encountered a number of spore-bearing organisms that are facultative in their relation to oxygen, and are therefore very difficult to eliminate; and there is, moreover, one in particular that, like the tetanus bacillus, forms a polar spore. This spore is, however, less round and much more oval than that of the tetanus bacillus, and gives to the organism containing it more the shape of a javelin (or *clostridium*, properly speaking) than that of a *pin*, the characteristic shape of the spore-bearing tetanus organism. It is non-pathogenic, and grows both with and without oxygen, and should, consequently, not be mistaken for the latter bacillus. It must also be borne in mind that there are occasionally present in the soil still other bacilli which form polar spores, and which, when in this stage, are almost identical in appearance with the tetanus bacillus; but they will usually be found to

differ from it in their relation to oxygen, and they are also without disease-producing properties.

MORPHOLOGY.—It is a slender rod with rounded ends. It may appear as single rods, or, in cultures, as long threads. It is motile, though not actively so. The motility is rendered somewhat more conspicuous by examining the organism upon a warm stage.

FIG. 94.



Bacillus tetani. A. Vegetative stage. B. Spore-stage, showing pin-shapes.

At the temperature of the body it rapidly forms spores. These are round, thicker than the cell, and usually occupy one of its poles, giving to the rod the appearance of a small pin. (Fig. 94.) When in the spore-stage it is not motile.

It is stained by the ordinary aniline staining-reagents. It retains the color when stained by Gram's method.

CULTURAL PECULIARITIES.—It is an obligate anaërobe, and cannot be brought to development under access of oxygen. It thrives in an atmosphere of pure hydrogen, but not in one of carbonic acid.

It grows in ordinary nutrient gelatin and agar-agar of a slightly alkaline reaction. Gelatin is slowly liquefied, with the coincident production of a small amount of gas. Neither agar-agar nor blood-serum is liquefied by its growth.

FIG. 95.

Colonies of the tetanus bacillus four days old, made by distributing the organisms through a tube nearly filled with glucose-gelatin. Cultivation in an atmosphere of hydrogen. (From FRÄNKEL and PFEIFFER.)

The addition to the media of from 1.5 to 2 per cent. of glucose, 0.1 per cent. of indigo-sodium sulphate, or 5 per cent. by volume of blue litmus tincture favors its growth.

It grows well in alkaline bouillon under an atmosphere of hydrogen.

Under artificial conditions it may be cultivated through numerous generations without loss of virulence.

APPEARANCE OF THE COLONIES.—Colonies of *bacillus tetani* on gelatin under an atmosphere of hydrogen have, in their early stages somewhat the appearance of the colonies of the common *bacillus subtilis* in their earliest stages, viz., they have a dense, felt-like centre surrounded by a fringe of delicate radii. The liquefaction is so slow that the appearance is retained for a relatively long time, but eventually becomes altered. In very old

colonies the entire mass is made up of a number of distinct threads that give it the appearance of a common mould. (See Fig. 95.)

In *stab-cultures* made in tubes about three-quarters filled with gelatin growth begins at about 1.5 to 3 cm. below the surface, and gradually assumes the appearance of a cloudy, linear mass, with prolongations radiating into the gelatin from all sides. Liquefaction with coincident gas-production results, and may reach almost to the surface of the gelatin.

Relation to Temperature and to Chemical Agents.—It grows best under a temperature of from 36° to 38° C.; gelatin cultures kept at from 20° to 25° C. begin to grow after three or four days. In an atmosphere of hydrogen at from 18° to 20° C. growth does not usually occur before one week. No growth occurs below 14° C. At the temperature of the body spores are formed in cultures in about thirty hours, whereas in gelatin cultures at from 20° to 25° C. they do not usually appear before a week, when the lower part of the gelatin is quite fluid.

Spores of the tetanus bacillus when dried upon bits of thread over sulphuric acid in the desiccator and subsequently kept exposed to the air, retain their vitality and virulence for a number of months. Their vitality is not destroyed by an exposure of one hour to 80° C.; on the other hand, an exposure of five minutes to 100° C. in the steam sterilizer kills them. They resist the action of 5 per cent. carbolic acid for ten hours, but succumb when exposed to it for fifteen hours. In the same solution, plus 0.5 per cent. of hydrochloric acid, they are no longer active after two hours. They are killed when acted upon for three hours by corrosive

duced by the bacteria *in the culture* from which they are obtained and introduced with them into the tissues of the animal at the time of inoculation. In support of the latter hypothesis: mice have been inoculated with pure cultures of this organism; after one hour the point at which the inoculation was made was excised and the tissues cauterized with the hot iron; notwithstanding the short time during which the organisms were in contact with the tissues and the subsequent radical treatment, the animals died after the usual interval and with the typical symptoms of tetanus.

The poison produced by the tetanus bacillus, and to which the symptoms of the disease are due, has been isolated and subjected to detailed study; some of its peculiarities, as given by Kitasato, are as follows:[1]

"When cultures of this organism are robbed of their bacteria by filtration through porcelain the filtrate contains the soluble poison, and is capable, when injected into animals, of causing tetanus.

"Inoculations of other animals with bits of the organs of the animal dead from the action of the tetanus poison produce no result; but similar inoculations with the blood or with the serous exudate from the pleural cavity always result in the appearance of tetanus. The poison is, therefore, largely present in the circulating fluids.

"The greatest amount of poison is produced by cultivation in fresh neutral bouillon of a very slightly alkaline reaction.

"The activity of the poison is destroyed by an ex-

[1] Zeitschrift für Hygiene, 1891, Bd. x. S. 267.

posure of one and one-half hours to 55° C.; of twenty minutes to 60° C.; and of five minutes to 65° C.

"By drying at the temperature of the body under access of air the poison is destroyed; but by drying at the ordinary temperature of the room, or at this temperature in the desiccator over sulphuric acid, it is not destroyed.

"Diffuse daylight diminishes the intensity of the poison. Its intensity is preserved for a much longer time when kept in the dark.

"Direct sunlight robs it of its poisonous properties in from fifteen to eighteen hours.

"Its activity is not diminished by diluting a fixed amount with water or nutrient bouillon.

"Mineral acids and strong alkalies lessen its intensity."

The chemical nature of this poison is not positively known, but according to the observations of Brieger and Cohn[1] it is not to be classed with the albumins in the sense in which the word is commonly used. When obtained in a pure, concentrated form its toxic properties are seen to be altered by acids, by alkalies, by sulphuretted hydrogen, and by temperatures above 70° C. Even when carefully protected from light, moisture, and air it gradually becomes diminished in strength. When freshly prepared by the methods of the authors just cited its potency is almost incredible, 0.00005 milligramme being sufficient to cause fatal tetanus in a mouse weighing fifteen grammes.

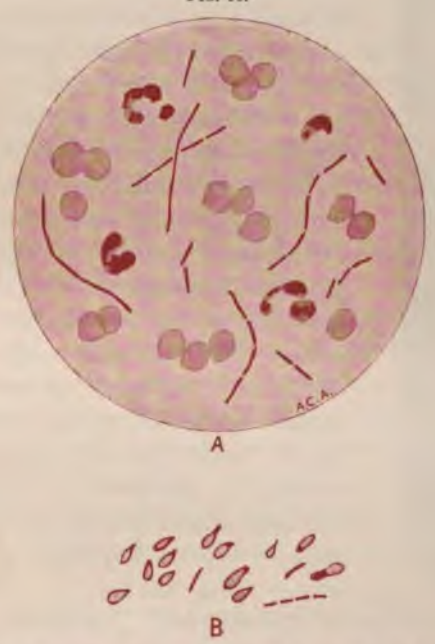
THE BACILLUS OF MALIGNANT ŒDEMA.

The bacillus of malignant œdema, also known as *vibrion septique*, is another pathogenic form almost

[1] Zeit. für Hygiene und Infektionskrankheiten, 1893, Band. xv. S. 1.

everywhere present in the soil. In certain respects it is a little like the bacillus of anthrax, and was at one time confounded with it; but it differs in the marked peculiarity of being a strict anaërobe. It was first observed by Pasteur, but it was not until later that Koch, Liborious, Kitt, and others described its peculiarities in detail. It can usually be obtained by

FIG. 96.



Bacillus œdematis maligni. A. Œdema-fluid, from site of inoculation of guinea-pig, showing long and short threads. B. Spore-formation, from culture.

inserting under the skin of rabbits or guinea-pigs small portions of garden-earth, street-dust, or decomposing organic substances. There results a widespread

œdema, with more or less gas-production in the tissues. In the œdematous fluid about the site of inoculation the organism under consideration may be detected. (Fig. 96, A.)

Fig. 97.

Colonies of the bacillus of malignant œdema in deep gelatin culture. (After Fränkel and Pfeiffer.)

It is a rod about 3 to 3.5 μ long and from 1 to 1.1 μ thick—*i. e.*, it is about as long as *bacillus anthracis*, but is a trifle more slender. It is usually found in pairs, joined end to end, but may occur as longer threads; particularly is this the case in cultures. When in pairs the ends that approximate are squarely cut, while the distal extremities are rounded. When occurring singly both ends are rounded. (How does it differ in this respect from *bacillus anthracis?*) It is slowly motile, and its flagella are located both at the ends and along the sides of the rod. It forms spores that are usually located in or near the middle of the cells, causing frequently a swelling at the points at which they are located and giving to the cell a more or less oval, spindle, or lozenge shape. (Fig. 96, B.)

It is an obligate anaërobe, growing on all the ordinary media, but not with access of oxygen. It grows well in an atmosphere of hydrogen. It causes liquefaction of gelatin.

In tubes containing about 20 to 30 c.c. of gelatin that has been liquefied, inoculated with a small amount

of the culture, and then rapidly solidified in ice-water, growth appears in the form of isolated colonies at or near the bottom of the tube in from two to three days at 20° C. These colonies, when of from 0.5 to 1 mm. in diameter, appear as spheres filled with clear liquid, and are difficult, for this reason, to detect. (Fig. 97.) As they gradually increase in size the contents of the spheres become cloudy and marked by fine radiating stripes, easily to be detected with the aid of a small hand-lens. In deep stab-cultures in agar-agar and in gelatin development occurs only along the track of puncture, at a distance below the surface. Growth is frequently accompanied by the production of gas-bubbles.

It causes rapid liquefaction of blood-serum, with production of gas-bubbles, and in two or three days the entire medium may have become converted into a yellowish, semi-fluid mass.

The most satisfactory results in the study of the colonies are obtained by the use of plates of nutrient agar-agar kept in a chamber in which all oxygen has been replaced by hydrogen. The colonies appear as dull whitish points, irregular in outline, and when viewed with a low-power lens are seen to be marked by a network of branching and interlacing lines that radiate in an irregular way from the centre toward the periphery.

It grows well at the ordinary temperature of the room, but reaches its highest development at the temperature of the body.

It stains readily with the ordinary aniline dyes. It does not stain by Gram's method.

PATHOGENESIS.—The animals known to be susceptible to inoculation with this organism are man, horses,

calves, dogs, goats, sheep, pigs, chickens, pigeons, rabbits, guinea-pigs, and mice. Cases are recorded in which men and horses have developed the disease after injuries, doubtless due to the introduction into the wound, at the time, of soil or dust containing the organism.

If one introduce into a pocket beneath the skin of a susceptible animal about as much garden-earth as can be held upon the point of a penknife, the animal frequently dies in from twenty-four to forty-eight hours. The most conspicuous result found at autopsy is a widespread œdema at and about the site of inoculation. The œdematous fluid is in some places clear, while at others it may be stained with blood; it is usually rich in bacilli (Fig. 94, A) and contains gas-bubbles. Of the internal organs only the spleen shows much change. It is large, dark in color, and contains numerous bacilli. If the autopsy be made immediately after death, bacilli are rarely found in the blood of the heart; but if deferred for several hours, the organisms will be found in this locality also, a fact that speaks for their multiplication in the body after death. At the moment of death they are present in varying numbers in all the internal viscera and on the serous surfaces of the organs.

Of all animals mice are probably the most susceptible to the action of this organism, and it is not rare to find it in the heart's blood, even immediately after death. They die, as a result of these inoculations, in from sixteen to twenty hours.

When a pure culture is used for inoculation a relatively large amount must be employed, and this should be introduced into a deep pocket in the subcutaneous tissues

some distance from the surface. In continuing the inoculations from animal to animal small portions of organs or a few drops of the œdema-fluid should be used. The inoculation may also be successfully made by introducing into a pocket in the skin bits of sterilized thread or paper upon which cultures have been dried.

The methods for obtaining the organism in pure culture, from the cadaver of an animal that has succumbed to infection by the bacillus of malignant œdema, are in all essential respects the same as those given for obtaining cultures from tissues in general; but it must be remembered that the organism is a strict anaërobe, and will not grow under the influence of oxygen. (See methods of cultivating anaërobic species.)

In certain superficial respects this bacillus suggests *bacillus anthracis*, but differs from it in so many important details that there is no excuse for confounding the two.

NOTE.—From what has been said of this organism, what are the most important differential points between it and *bacillus anthracis?* Inoculate several mice with small portions of garden-earth and street-dust. Isolate the organism that agrees most nearly with the description here given for the bacillus of malignant œdema. Compare its morphological, biological, and pathogenic peculiarities with those of *bacillus anthracis* under similar circumstances; especially its action on animals and its appearance in the tissues and fluids.

Still another pathogenic organism that may be present in the soil is

THE BACILLUS OF SYMPTOMATIC ANTHRAX; BACTÉRIE DU CHARBON SYMPTOMATIQUE (FR.); BACILLUS DES RAUSCHBRAND (GER.).

It is the organism concerned in the production of the disease of young cattle and sheep commonly known as "black leg," "quarter evil," and "quarter ill," a disease that prevails in certain localities during the warm months, and which is characterized by a peculiar emphysematous swelling of the muscular and subcutaneous cellular tissues over the quarters. The muscles and cellular tissues at the points affected are seen on section to be saturated with bloody serum, and the muscles particularly are of a dark, almost black color. In these areas, in the bloody transudates of the serous cavities, in the bile, and, after death, in the internal organs, the organism to be described can always be detected. It is manifest from this that the soil of localities over which infected herds are grazing may readily become contaminated through a variety of channels, and thus serve as a source of further dissemination of the disease.

The organism was first observed by Feser, and subsequently by Bollinger and others. The most complete description of its morphological and biological peculiarities is that of Kitasato.[1] The following is from Kitasato's contributions: it is an actively motile rod about 3 to 5 μ long by 0.5 to 0.6 μ thick. It has rounded ends, and, as a rule, is seen singly, though now and then pairs joined end to end may occur. It has no tendency to form very long threads. (Fig. 98, A.)

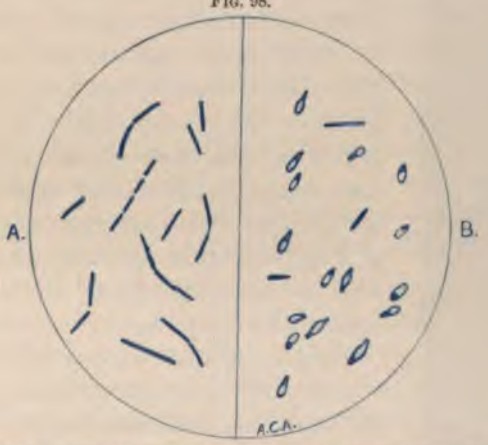
It forms spores, and when in this stage is seen to be

[1] Zeitschrift für Hygiene, Bd. vi. S. 105; Bd. viii. S. 55.

slightly swollen at or near one of its poles, the location in which the spore usually appears. (Fig. 98, B.) It is markedly prone to undergo degenerative changes, and involution-forms are commonly seen, not only in fresh cultures, but in the tissues of affected animals as well.

Though actively motile when in the vegetative stage it, like all other motile spore-forming bacilli, loses this

FIG. 98.



Bacillus of symptomatic anthrax. A. Vegetative stage—gelatin culture. B. Spore-forms—agar-agar culture.

property and becomes motionless when spores are forming.

It is strictly anaërobic and cannot be cultivated in an atmosphere in which oxygen is present. It grows best under hydrogen, and does not grow under carbonic acid.

The media most favorable to its growth are those containing glucose (1.5 to 2 per cent.), glycerin (4 to 5 per cent.), or some other reducing-body, such as indigo-sodium sulphate, sodium formate, etc.

When cultivated upon gelatin plates in an atmosphere

of hydrogen the colonies appear as irregular, slightly lobulated masses. After a short time liquefaction of the gelatin occurs and the colony presents a dark, dense, lobulated and broken centre, surrounded by a much more delicate, fringe-like zone.

FIG. 99.

Colonies of the bacillus of symptomatic anthrax, in deep gelatin culture. (After FRÄNKEL and PFEIFFER.)

When distributed through a deep layer of liquefied gelatin that is subsequently solidified colonies develop at only the lower portions of the tube. The single colonies appear as discrete globules that cause rapid liquefaction of the gelatin, and ultimately, coalesce into irregular, lobulated, liquid areas. In some of the larger colonies an ill-defined, concentric arrangement of alternate clear and cloudy zones can be made out. (Fig. 99.)

In deep stab-cultures in gelatin growth begins after about two or three days at 20° to 25° C. It begins usually at about one or two centimetres below the surface, and causes slow liquefaction at and around the track of its development. During its growth gas-bubbles are produced.

In deep stab-cultures in agar-agar at 37° to 38° C. growth begins in from twenty-four to forty-eight hours, also at about one or two centimetres below the surface, and is accompanied by the production of gas-bubbles. There is produced at the same time a peculiar, penetrating odor somewhat suggestive of that of rancid

butter. Under these conditions spores are formed after about thirty hours.

It grows well in bouillon of very slightly acid reaction under hydrogen, but does not retain its virulence for so long a time as when cultivated upon solid media. In this medium it develops in the form of white flocculi that sink ultimately to the bottom of the glass and leave the supernatant fluid quite clear. If the vessel be now gently shaken, these delicate flakes are distributed homogeneously through it. In bouillon cultures there is often seen a delicate ring of gas-bubbles round the point of contact of the tube and the surface of the bouillon. There is produced also a peculiar, penetrating, sour or rancid odor.

It grows best at the body-temperature—*i. e.*, from 37° to 38° C.—but can also be brought to development at from 16° to 18° C. Below 14° C. no growth is seen. Spore-formation appears much sooner at the higher than at the lower temperatures. When its spores are dried upon bits of thread in the desiccator over sulphuric acid, and then kept under ordinary conditions, they retain their vitality and virulence for many months. Similarly, bits of flesh from the affected areas of animals dead of this disease, when completely dried, are seen to retain for a long time the power of reproducing the disease. The spores are tolerably resistant to the influence of heat: when subjected to a temperature of 80° C. for one hour their virulence is not affected, but an exposure to 100° C. for five minutes destroys them. They are also seen to be somewhat resistant to the action of chemicals: when exposed to 5 per cent. carbolic acid they retain their disease-producing properties for about ten hours, whereas the vegetative forms

are destroyed in from three to five minutes; in corrosive sublimate solution of the strength of 1 : 1000 the spores are killed in two hours.

When gelatin cultures are examined microscopically the organisms are usually seen as single rods with rounded ends. When cultivated in agar-agar at a higher temperature spores are formed after a short time; the spores are oval, slightly flattened on their sides, thicker than the bacilli, and, as stated, frequently occupy a position inclining to one of the poles of the bacillus, though they are as often seen in the middle.

Bacilli containing spores are usually clubbed or spindle shape.

This bacillus stains readily with the ordinary aniline dyes. It is decolorized by Gram's method. Its spores may be stained by the methods usually employed in spore-staining.

Pathogenesis.—When susceptible animals, especially guinea-pigs, are inoculated in the deeper subcutaneous cellular tissues with pure cultures of this organism, or with bits of tissue from the affected area of another animal dead of the disease, death ensues in from one to two days. It is preceded by rise of temperature, loss of appetite, and general indisposition. The site of inoculation is swollen and painful, and drops of bloody serum may sometimes be seen exuding from it. At autopsy the subcutaneous cellular tissues and underlying muscles present a condition of emphysema and extreme œdema. The œdematous fluid is often blood-stained and the muscles are of a blackish or blackish-brown color. The lymphatic glands are markedly hyperæmic. The internal viscera present but little alteration visible to the naked eye. In the blood-

stained serous fluid about the point of inoculation short bacilli are present in large numbers. These often present slight swellings at the middle or near the end. They are not seen as threads, but lie singly in the tissues. Occasionally two will be seen joined end to end. If the autopsy be made immediately after death, these organisms may not be detected in the internal organs; but if not made until after a few hours, they will be found there also. In recent autopsies only vegetative forms of the organism may be found; but later (in from twenty to twenty-four hours) spore-bearing rods may be detected. (How does this compare with *bacillus anthracis?*) By successive inoculations of susceptible animals with serous fluid from the site of inoculation of the dead animal the disease may be reproduced.

Cattle, sheep, goats, guinea-pigs, and mice are susceptible to infection with this organism, and present the conditions above described; whereas horses, asses, and white rats present only local swelling at the site of inoculation. Swine, dogs, cats, rabbits, ducks, chickens, and pigeons are, as a rule, naturally immune from the disease.

Though closely simulating the bacillus of malignant œdema in many of its peculiarities, this organism can, nevertheless, be readily distinguished from it. It is smaller; it does not develop into long threads in the tissues; it is more actively motile, and forms spores more readily in the tissues of the animal than does the bacillus of malignant œdema. In their relation to animals they also differ; for instance, cattle, while conspicuously susceptible to symptomatic anthrax, are practically immune from malignant œdema; and while swine, dogs, rabbits, chickens, and pigeons are readily infected

with malignant œdema, they are not, as a rule, susceptible to symptomatic anthrax Horses are affected only locally, and not seriously, by the bacillus of symptomatic anthrax; but they are conspicuously susceptible to both artificial inoculation and natural infection by the bacillus of malignant œdema.

The distribution of the two organisms over the earth's surface is also quite different. The œdema bacillus is present in almost all soils, while the bacillus of symptomatic anthrax appears to be confined to certain localities, especially places over which infected herds have been pastured.

A single attack of symptomatic anthrax, if not fatal, affords subsequent protection; while infection with the malignant œdema bacillus appears to predispose to recurrence of the disease. (Baumgarten.)

CHAPTER XXVI.

Infection and immunity—The types of infection; intimate nature of infection—Septicæmia, toxæmia, variations in infectious processes—Immunity, natural and acquired—The hypotheses that have been advanced in explanation of immunity—Conclusions.

An organism capable of producing disease we call *pathogenic* or *infective*, and the process by which it produces disease we know as *infection*. Diseases, therefore, that depend for their existence upon the presence of bacteria in the tissues are *infectious* diseases.

What is the intimate nature of this process we call infection? Is it due to the *mechanical presence* of living bacteria in the body, or does it result from the *deposition* in the tissues of substances produced by these bacteria that are either locally or generally incompatible with life? Or, is the group of pathological alterations and constitutional symptoms seen in these diseases the result of *abstraction from* the tissues, by the bacteria growing in them, of substances essential to their vitality? These are some of the more important of the questions that present themselves in the course of analysis of this interesting phenomenon.

Let us look into several typical infectious diseases, note what we find, and see to what extent the observations thus made will aid us in formulating an opinion. We begin with a study of those diseases in which there is a general infection—*i. e.*, in which there is a general distribution of the infective agents throughout the body.

This group comprises the "septicæmias," and of them the disease of animals known as anthrax represents a type of the condition. If the cadaver of an animal dead of anthrax be examined by bacteriological methods, there will be discovered present in all the organs and tissues an organism, a bacillus, of definite form and biological characteristics; and if the organs, and tissues generally, be subjected to microscopic examination this same organism will be found always present and always located within the capillaries. At many points it will be seen crowded in the capillaries in such numbers as almost, if not quite, to burst them, and very commonly their lumen for a considerable extent is entirely occluded by the growing bacilli. In such a case as this we might be tempted to conclude that death had resulted from mechanical interference with the capillary circulation. Suppose, however, we subject the cultures obtained from this animal to conditions, either chemical or thermal, that are not particularly favorable to their normal development, and from time to time inoculate susceptible animals with the cultures so treated. The result will be that, as we continue to expose our cultures to unfavorable surroundings, the period of time that is required for them to cause the death of animals will, in some cases, gradually become extended, until finally death will not ensue at all after inoculation. If, as these animals die, a careful record of the conditions found at autopsy be kept and compared, it will ultimately be noticed that the animals that die at a longer time after inoculation present conditions more or less at variance with those seen in the original animal that died more quickly after having been inoculated. These differences usually consist in a diminution of the num-

ber of bacilli that appear upon culture plates from the blood and internal organs, and in a lessening in the amount of mechanical obstruction offered to the circulation through plugging of the capillaries by masses of bacilli, as detected by microscopic examination of sections of the organs; indeed, this latter condition may often have almost, if not quite, disappeared. We see here an animal dead from the invasion of the same organism that produced death in the first animal, but with little or none of those particular lesions to which we were inclined to attribute the death of that animal. It is apparent, then, that this organism with which we have been working can destroy the vitality of an animal in a way other than by mechanically obstructing its blood-vessels; it possesses some other means of destroying life. Possibly its growth in the tissues is accompanied by the production of soluble poisons, which when present in the blood are not compatible with life.

Let us see if the study of another group of infections will furnish any evidence in support of such an hypothesis. Introduce into the subcutaneous tissues of a guinea-pig a small amount of a pure culture of the bacillus of diphtheria. In three or four days the animal dies. We proceed with our autopsy in exactly the same way that we did with the animals dead of anthrax, and are astonished to find that the organs, blood, and tissues generally are sterile,[1] in so far as the presence of the organism with which the animal was inoculated is concerned, and by both culture and microscopic methods it is possible to detect them only at the site of inoculation, where they were deposited. It is very evident

[1] In by far the greater number of cases this is true; but under particular circumstances there are exceptions.

that we have here a condition with which mechanical plugging of the capillaries could have had no connection, for there are no organisms in the blood to interfere with its circulation. Our hypothesis then with regard to the condition found in our first case of anthrax is again not tenable. Similarly, if an animal that has died of tetanus be examined, we do not find the bacilli in the tissues and circulating fluids generally, and, indeed, often fail to find them at the point of injury. Plainly, the fatal results following upon inoculations with the diphtheria and the tetanus bacillus, with their accompanying tissue-changes, occur from the presence of a something that cannot be detected by either cultural or microscopic methods, and this something can be only a soluble substance that is produced by the growing bacteria at the site of inoculation, gains access to the circulation, and through this channel causes death, for it is scarcely to be imagined that the insignificant wound made in the course of inoculation could *per se* have had this effect. In other words, these latter animals have died from what is called *toxæmia* (poison in the blood), a condition distinctly different from *septicæmia*, as seen in our first animal dead of anthrax.

There are, again, other infectious diseases, many of which are known to present variations from what might be considered a typical course, that may still further serve to support the view that infection is a process in which the mechanical effect of organisms in the circulating fluids is of little consequence. Conspicuous among these are the infections that follow upon the introduction into the tissues of susceptible animals of cultures of *micrococcus lanceolatus* (pneumococcus), of the bacillus of chicken cholera, and of the organisms con-

cerned in the production of the so-called "hemorrhagic septicæmias." When running their normal course the specific organisms of these diseases cause typical septicæmias in susceptible animals; but often, from causes not entirely clear, the animals die with only local lesions, or with but very few organisms in the internal viscera. We see here conditions analogous to those observed in the two experiments with anthrax, viz., we find a group of diseases that are properly classed as septicæmias, because of the usual general invasion of the body by the organisms concerned in their production, but which frequently assume a purely local character—in both instances proving fatal to the animal infected. From what we have seen it is manifestly probable that, whether these diseases be designated as septicæmias or as toxæmias, death is produced in all instances by poisonous substances that are generated by the infecting bacteria. In the case of typical anthrax, and other varieties of septicæmia, the production of this poison is associated with the general dissemination of the organisms throughout the body; while in those infections often referred to as toxæmias, of which diphtheria may be taken as a type, the poison is produced by the organisms that remain localized at the site of invasion, and is thence disseminated throughout the body by the circulating fluids. Infection thus far, then, appears to be a chemical process.

Through special investigations that have been made upon the products of growth of certain pathogenic bacteria this opinion receives further confirmation; it has been found possible by the use of appropriate methods to isolate from among the mass of material in which certain of these organisms have been artificially culti-

vated substances which, when separated from the bacteria by which they were produced, possess the power of causing in animals all the constitutional symptoms and pathological tissue-changes that occur in the course of infection by the organisms themselves. In some instances these poisons—toxins,[1] as they are collectively called—appear to be the direct result of metabolic changes brought about by bacteria in the medium or tissues in which they may be developing—*i. e.*, they are products of nutrition that pass readily into solution, as is conspicuously seen in the case of the bacillus of diphtheria and of tetanus when under both artificial cultivation and in the animal body. Many bacteria which do not possess the power of generating or secreting such poisons may, nevertheless, have intimately associated with their protoplasmic bodies poisonous substances that can only be isolated by particular methods; thus the toxins of *bacillus tuberculosis* and of *spirillum choleræ Asiaticæ* are much more conspicuously present in the protoplasm of these bacteria than in the fluids in which they have grown; and Buchner has isolated from several species of bacteria "bacterio-proteins" having the common properties of solubility in alkalies, resistance to the boiling temperature, attraction of leucocytes (positive chemotaxis[2]), and pyogenic powers.

There is as yet little agreement of opinion as to the chemical nature of toxins; but it is probable that the

[1] The term "toxins" is commonly applied to *amorphous*, nitrogenous poisons produced by bacteria in both living tissues and dead substances; while, on the other hand, "ptomaïns" relates to *crystallizable*, nitrogenous poisons that are formed in dead tissue, and "leucomains" to poisonous and non-poisonous alkaloidal bodies that occur in living tissues as a result of physiological metabolism.

[2] See Chemotaxis.

group comprises different bodies of the nature of globulins, nucleo-albumins, peptones, albumoses, and enzymes or ferments.

Toxic ptomaïns are probably not conspicuously concerned in producing the characteristic symptoms of infection, as they are absent from cultures of certain highly pathogenic bacteria.

In some instances the production of poisonous principles, even under artificial conditions of cultivation, is most astonishing, and poisons result that in the degree of their toxicity exceed anything hitherto known to us. For instance, the potencies of the poisons that have been isolated from cultures of *bacillus diphtheriæ* and of the bacillus of tetanus have been carefully determined by experiments upon animals, and it has been found that 0.4 milligramme of the former is capable of killing eight guinea-pigs, each weighing 400 grammes, or two rabbits, each weighing 3 kilogrammes (Roux and Yersin[1]); and that 0.0001 milligramme of the latter will produce tetanus in a mouse, with all the characteristic manifestations of the disease (Brieger and Cohn[2]).[3]

Briefly stated, the invasion of the body by infective micro-organisms may best be conceived as a contest between the invading organisms on the one side and the resisting tissues of the animal body on the other; the weapons of offence of the former being the poisonous products of their growth, while the means of defence possessed by the latter are vital substances

[1] Annales de l'Institut Pasteur, 1889, tome iii. p. 287.

[2] Zeitschrift für Hygiene und Infektionskrankheiten, 1893, Bd. xv. Heft 1.

[3] By the use of more recently devised methods we are enabled to increase still further the toxicity of these poisons; especially is this the case with regard to the diphtheria toxin.

which act, so to speak, as antidotes to these poisons. If the tissue-elements are not of sufficient vigor to destroy the invading bacteria or to render inert the poisons produced by them, the bacteria are victorious and infection results; on the other hand, if there be failure to excite a condition of disease, the tissues are victorious, and are then said to be *resistant* to or *immune* from this particular form of infection.

In some cases the animal antidotes act directly upon the invading organisms themselves—*i. e.*, they are germicidal; in others their function is more that of antidotes, or neutralizers in the chemical sense, for the poisons produced by these organisms, the organisms themselves, in certain instances, experiencing only slight injury from a limited sojourn in the living tissues. To the substances possessing the germicidal functions that are normally present in the animal body Buchner gives the name "alexins," and Hankin "defensive proteids;" while to those ill-defined elements of the body that act more directly upon the soluble bacterial poisons the name "antitoxins" is now generally applied. Unlike the alexins of Buchner and the defensive proteids of Hankin, it is only occasionally that antitoxic activities are demonstrable in normal living tissues as a congenital characteristic;[1] in the majority of cases they are conspicuously present only as an acquired peculiarity. Our acquaintance with these bodies extends little further than their physiological functions and some of the means that induce their

[1] See Bolton: Transactions of Association of American Physicians, 1896, vol. xi. p. 62. Pfeiffer: Deutsche med. Wochenschrift, 1896, No. 8. Fischl and v. Wunschheim: Centralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten, 1896, Abt. i. Bd. xix. S. 652. Wassermann: Berliner klin. Wochenschrift, 1898, No. 1.

generation. We have no satisfactory knowledge of their intimate nature or of the primary sources of their production. They are believed by some (Buchner[1] and Metchnikoff[2]) to represent bacterial toxins that have been converted through the vital activity of the integral cells of the body; and Roux[3] and Buchner[4] hold that they exhibit their protective functions less by direct, destructive combination with the toxins than by a specific stimulation of the tissue-cells that enables the latter to resist the harmful influences of the poisonous bacterial products. On the other hand, Behring,[5] Ehrlich,[6] and their associates contend that they are vital tissue-elements ("toxophores," Ehrlich), having the property of combining directly with the toxins to form "physiologically neutral" toxin-antitoxin compounds that are in a manner analogous to the double salts of certain chemical reactions.

It is a common observation that many human beings and animals are less susceptible to the different forms of infection than are others; and that particular species of animals not only do not suffer naturally from certain specific diseases, but resist all efforts to produce the diseases in them by artificial methods; in other words, they are *naturally immune* from them. The term "natural immunity," as now employed, implies a congenital condition of the individual or species, a condition peculiar to his idioplasm,

[1] Buchner: Münchener med. Wochenschrift, 1893, Nos. 24 and 25.
[2] Metchnikoff: Weil's Handbuch der Hygiene, Bd. ix. Lieferung 1.
[3] Roux: Annales de l'Institut Pasteur, 1894, p. 722.
[4] Buchner: Berliner klin. Wochenschrift, 1894, No. 4.
[5] Behring: Infektion und Desinfektion, Leipzig, 1894, S. 248.
[6] Ehrlich: Klinisches Jahrbuch, 1897, Bd. vi. Heft 2, S. 311. Fortschritte der Medicin, 1897, Bd. xv. No. 2.

which has been transmitted to him as a tissue-characteristic through generations of progenitors.

Again, it is often observed that an individual or an animal after having recovered from certain forms of infection has thereby acquired protection from subsequent attacks of like character; in other words, they are said to have *acquired immunity* from this disease. "Acquired immunity" implies, therefore, a condition of the tissues of an individual, not of necessity peculiar to other members of the race or species, that has originated during his life from the stimulation of his integral cells by one or another of the specific, infective irritants that may have been purposely introduced, or accidentally gained access to his body.

The problem involving the explanation of the foregoing interesting ideas and observations has afforded material for reflection and hypothesis for a long time. It is only through investigations conducted during recent years that it has met with anything approaching satisfactory solution, and even now there remain a number of important points that are veiled in obscurity.

Conspicuous among the observers who have endeavored to explain the *modus operandi* of immunity may be mentioned Chauveau, Pasteur, Metchnikoff, Buchner, Flügge and his pupils (Smirnow, Sirotinin, Bitter, Nuttall), Fodor, Hankin, Pfeiffer, Ehrlich, Behring, Roux, and a host of others whose names are more or less prominent in current literature. In the following pages we will present briefly the result of investigations by these various authors.

In 1880 Chauveau [1] suggested an explanation for the phenomenon of immunity that has since been known as

[1] Comptes rendus, etc., July, 1880, No. 91.

the "*retention hypothesis.*" It is, in short, as follows: that the immunity commonly seen to exist in animals that have passed through an attack of infection from a subsequent outbreak of the same malady, and likewise the immunity that has been produced artificially by vaccination, exist by virtue of some bacterial product that has been *retained or deposited* in the tissues of those animals, and that this product by its presence prevents the development of the same organisms if they should subsequently gain access to the body.

Bearing upon this view the experiments of Sirotinin,[1] made with cultures of various pathogenic bacteria, demonstrated that, in so far as culture-experiments were concerned, the only substance produced by growing bacteria that could be in any way inimical to their further development were substances that gave rise to alterations in the reaction of the medium in which they were developing—*i. e.*, acids or alkalies produced by the bacteria themselves. So long as the organisms were not actually dead from exposure to these substances correction of the abnormal reaction was followed by further development of the organisms. Sirotinin also states that materials containing the products of growth of bacteria, so long as they are maintained at a neutral or only slightly alkaline reaction, serve very well as media upon which to cultivate again the same organism that produced them, providing the nutritive elements have not been entirely exhausted. He remarks that, if in such a concentrated form as we find the life-products of bacteria in the medium in which they are growing, no inhibitory compounds other than acids and alkalies are to be detected, it is hardly probable that they are produced

[1] Zeitschrift für Hygiene, 1888, Bd. iv.

in the tissues of the living animal, and retained there, to a degree sufficient to prevent the growth of bacteria that may subsequently gain entrance to these tissues, after the disappearance of the organisms concerned in the primary invasion. On the other hand, Salmon and Smith,[1] Roux and Chamberland,[2] and others had demonstrated that a sort of immunity against certain forms of infection may be afforded to susceptible animals by the injection into their tissues of the products of growth of particular organisms which would, if themselves introduced into the animal body, produce fatal results. Though this observation of Salmon and Smith attracted comparatively little attention at the time it was made, it serves, nevertheless, as we shall see subsequently, as the starting-point for a line of investigation that has furnished practically all the information of importance that we possess on this complicated subject.

As opposed to the view of Chauveau, Pasteur[3] and certain of his pupils believed that the immunity frequently afforded to the tissues by an attack of infection or following upon vaccination against infection, was due rather to an *abstraction* from the tissues, by the organisms that were concerned in the primary attack, of a something that is necessary to the growth of the infecting organism should it gain entrance to the body at any subsequent time. This view is known as the "*exhaustion hypothesis.*"

As to the exhaustion hypothesis of Pasteur, there is, as yet, no evidence whatever for its support; and, in

[1] Proceedings of the Biological Society, Washington, D. C., 1886, vol. iii.

[2] Annales de l'Institut Pasteur, 1888–'89, tomes i., ii.

[3] Bulletin de l'Académie de Médecine, 1880.

fact, in the light shed by more recent investigations this is probably the least tenable of any of the several hypotheses advanced in explanation of this most complicated phenomenon. The work of Bitter,[1] which was undertaken with the view of determining if, in the process of acquiring immunity, there occurred this exhaustion from the tissues of material necessary to the growth of bacteria that might gain entrance to them at some later date, gave only negative results. The flesh of animals in which immunity had been produced contained all the elements necessary for the growth and nutrition of the bacteria against which the animals had been protected, just as did the flesh of non-vaccinated animals.

1884 Metchnikoff[2] published the first of a series of observations upon the behavior of certain of the mesodermal cells of lower animals toward insoluble particles that may be present in the tissues of these animals. The outcome of these investigations was the establishment of his well-known doctrine of *phagocytosis*, the principle of which is that the wandering cells of the animal organism, the leucocytes, possess the property of taking up, rendering inert, and digesting micro-organisms which they may encounter in the tissues. Metchnikoff believed that in this way immunity from infection may in many, if not all, cases be explained. He believed that susceptibility to or immunity from infection was essentially a matter between the invading bacteria on the one hand, and the leucocytes of the tissues on the other. The suc-

[1] Zeitschrift für Hygiene, 1888, Bd. iv.

[2] Arbeiten aus dem Zoölogischen Institut der Universität Wien, 1884, Bd. v. Fortschritte der Medicin, 1884, Bd. ii.

cess or failure of the leucocytes in protecting the animal against infection depends, according to this doctrine, entirely upon the efficiency of the provisions possessed by them for destroying bacteria. When these means are of sufficient vigor to bring about the death of the bacteria the tissues are victorious; but when the poisons generated by the bacteria are potent to arrest the phagocytic action of the leucocytes then the tissues succumb and infection results.

Has this doctrine of phagocytosis, as advanced by Metchnikoff, stood the test of experimental criticism? Evidence that has accrued since the time of its suggestion has rendered questionable the advisability of its unconditional adoption in the sense that Metchnikoff propounded it. The later studies of a number of investigators indicate that while the leucocytes play a most important part in the phenomenon of immunity, it is hardly likely that this always occurs through their taking up within themselves and digesting invading bacteria, as Metchnikoff believes; but rather that their part in the process is to secrete protective chemical substances that are thrown into the circulating blood, and which, in part at least, comprise the defensive bodies to which Buchner has given the name "alexins."[1]

The first severe blow that Metchnikoff's theory of phagocytosis received was given by Nuttall,[2] in his work upon the bactericidal property of the animal economy. In these experiments Nuttall showed positively that the destruction of virulent bacteria in the blood of animals was not necessarily dependent upon the actual presence of living leucocytes, but that the serum

[1] See Hahn: Archiv für Hygiene, 1895, Bd. xxv. S. 105.

[2] Zeitschrift für Hygiene, 1888, Bd. iv.

of the blood when quite free from cellular elements possessed this power to a degree equal to that of the blood when all the constituent parts were present. In the blood, as such, phagocytosis could be seen; but, as a rule, the bacteria presented evidence of having undergone degenerative changes before they had been taken up by the wandering cells—*i. e.*, the bacteria had evidently been injured or killed by the fluids before they were attacked by the phagocytes.

Contrary to the beliefs in existence at the time, Traube and Gscheidlen,[1] as far back as 1874, demonstrated that considerable quantities of septic material could be injected into the circulation of warm-blooded animals without apparently any effect upon the animal. Particularly was this the case with dogs. If they injected into the circulation of a dog as much as 1.5 c.c. of decomposing fluid, blood drawn from the animal after from twenty-four to forty-eight hours showed no special tendency to decompose, though it was kept under observation for a long time. As a result of these experiments, the question that naturally presented itself was: Does the animal organism possess the power of rendering septic organisms inert, and if so, to what extent? They believed this power of rendering living organisms inert to be possessed by the circulating blood to only a limited degree, for after the injection of much larger amounts of the putrid fluid into the blood of the animal death usually ensued in from twenty-four to forty-eight hours. The blood drawn from the animal just before death contained the living bacteria of putrefaction and underwent decomposition. They attributed the germicidal phenomenon to the

[1] Jahresb. der Schlesischen Ges. für Cultur., 1874; Jahr. iii. p. 179.

action of the "ozonized oxygen of the corpuscles of the blood."

Similar observations were made in 1885 by Fodor,[1] who remarks upon the rapidity with which living bacteria disappear from the circulating blood of animals; and Wyssokowitsch,[2] who endeavored to explain this disappearance experimentally, went wide of the mark by concluding that they were filtered from the blood and digested by the parenchymatous tissues.

In 1882 Rauschenbach[3] demonstrated that when in the process of coagulation fibrin was formed, it was not as a specific product of the action of the colorless elements of the blood alone, but also as a result of the combined action between all animal protoplasms and healthy blood-plasma, and that in the process there was always a disintegration of the leucocytes present. In 1884 Groth[4] demonstrated further that such a disintegration of leucocytes occurred in normal circulating blood, though here it was not accompanied by coagulation. The results of these observations suggested the question: Does such a disintegration occur when *vegetable* protoplasm is introduced into the blood?

For the purpose of answering this question, Grohmann,[5] a pupil of Alexander Schmidt, undertook to study the action of the circulating blood upon the vegetable protoplasm of bacteria. He noticed that clotting of the blood

[1] Fodor: Archiv für Hygiene, Bd. iv. S. 129.

[2] Wyssokowitsch: Zeitschrift für Hygiene, 1886, S. 3.

[3] Ueber die Wechselwirkung zwischen Protoplasma und Blutplasma. Dissertation, Dorpat, 1882.

[4] Ueber die Schicksale der farblosen Elemente in kreisendem Blut. Dissertation, Dorpat, 1884.

[5] Ueber die Einwirkung des zellenfreien Blutplasma auf einige pflanzliche Mikro-organismen. Dissertation, Dorpat, 1884.

of the horse was very much accelerated by the addition to it of certain bacteria; that at the same time the development of the bacteria was checked, and in the case of the pathogenic varieties their virulence was diminished. This was particularly the case when the anthrax bacillus was employed.

Grohmann seems to have appreciated the significance of this observation, though he made no attempt to study the subject more closely. He remarks that the system probably possesses, in the plasma of the blood, a body having disinfectant properties.[1] His observations on this particular point were, however, only incidental, and it was not until the researches of Nuttall, directed especially toward the question of germicidal properties of normal animal fluids, that a complete and acceptable demonstration of this important function was forthcoming, and that a general interest, commensurate with its importance, was created in the subject.

Nuttall's results received confirmation from all sides. Fodor,[2] Buchner,[3] Lubarsch,[4] Nissen,[5] Stern,[6] Prudden,[7] Charrin and Roger,[8] and many others continued in the same line, and all made practically the same observation.

After the demonstration by Nuttall that the serum of the blood was directly detrimental to the vitality of certain pathogenic bacteria, a number of investigators undertook to determine the conditions most favorable to

[1] Loc. cit., pp. 6 and 33.

[2] Central. für Bakteriologie und Parasitenkunde, 1890, Bd. vii. No. 24.

[3] Archiv für Hygiene, 1890, vol. x. parts 1 and 2.

[4] Centralblatt für Bakteriologie und Parasitenkunde, 1889, Bd. vi. No. 18.

[5] Zeitschrift für Hygiene, 1889, Bd. vi. part 3.

[6] Zeitschrift für klin. Medicin, 1890, Bd. viii. parts 1 and 2.

[7] New York Medical Record, 1890, vol. xxxvii. pp. 85 and 86.

[8] Société de Biologie de Paris.

the exhibition of this phenomenon, and further to decide upon the constituent of the serum to which this property is due, or if it is a function of the serum only as a whole.

In the course of Buchner's experiments it was demonstrated that the serum was robbed of this property by exposure to a temperature of 55° C. for half an hour; that its efficacy as a germicide was not diminished by alternate freezing and thawing; that by dialysis or extreme dilution with distilled water its germicidal activity was diminished or completely checked; but that an equal dilution could be made, if sodium chloride solution (0.6–0.7 per cent.) was substituted for the distilled water, without a reduction of its bactericidal activity. From this he concluded that the active element in this phenomenon is a living albumin, an essential constituent of which is sodium chloride, and which, when robbed of this salt either by dialysis or dilution, becomes inert in its behavior toward bacteria. For this or these germicidal constituents of the blood he suggested the name "alexins."

Buchner found, moreover, that the activity of the serum alone against bacteria was greater than when the cellular elements of the blood were present. This he explains by the assumption that in the serum alone the germicidal element predominates, whereas in the blood, as such, outside of the body, it is still present, but its influence is counteracted by the nutrition offered to the bacteria by the disintegrated cellular elements; so that here the nutritive feature is most conspicuous and the destructive activity toward bacteria is less effectual.

A closer study of the nature of this germicidal element in the body of animals was made by Hankin and

Martin.[1] The former isolated from the spleen and lymphatic glands a body—a globulin—which in solution possesses germicidal properties.

Similar germicidal, ferment-like globulins have been isolated from the blood by Ogata,[2] and in their studies upon tetanus Tizzoni and Cattani[3] found a body that was antagonistic to the poison produced by the organism of this disease.

In 1890 Fodor[4] showed that the fatal results of anthrax infection could be materially retarded by alkalinization of the circulating blood; and he further states that it is occasionally possible by the administration of alkalies *per os* to rescue animals already infected.

According to the observations of Vaughan,[5] the most important germicidal or protective agents possessed by the body are the nucleins; and Kossel has shown that the cholera vibrio, streptococcus, staphylococcus, and typhoid bacillus are destroyed by 0.5 per cent. solution of nucleinic acid.

Hankin believes the globulins or "defensive proteids" that he has discovered and the albuminoid bodies studied by Buchner to be identical. The most interesting and, in the light of work that has appeared since, the most important, of Hankin's observations were not those upon the power of these globulins to destroy the vitality of living organisms, but rather those upon the relation between them and the poisonous metabolic products of growth of the organisms.

[1] British Medical Journal, May 31, 1890.

[2] Centralblatt für Bakteriologie und Parasitenkunde, 1891, vol. ix. S. 599.

[3] Ibid., p. 685.

[4] Fodor: Centralblatt für Bakteriologie, 1890, Bd. vii. S. 753–766.

[5] Vaughan: Medical News, May 20, 1893.

For example, if the poisonous products of *virulent* anthrax bacilli be isolated and mixed with the globulin extracted from normal tissues, the experiments of Hankin showed a directly destructive action on the part of the bacterial products. He found that the amount of poisonous albumose produced by the *attenuated* anthrax bacilli employed as vaccines was much less than that produced by organisms possessing full virulence; and he suggests that perhaps the protective influence of vaccinations that are practised by introducing into the animal the organisms that have been attentuated in virulence is due to a gradual tolerance acquired by the cells of the tissues to the action of the poison when produced in these small quantities; in the same way that a tolerance was acquired by the tissues for the venom of the rattlesnake in the experiments of Sewall[1] (and more recently in the work of Fraser,[2] Calmette, Weir Mitchell, and others), and similar to that following the injection into the tissues of small quantities of hemialbumose, which in large amounts rapidly proves fatal.

Of utmost importance to these investigations upon the blood and fluids of the body are the experiments

[1] Journal of Physiology, 1887, vol. vii. p. 203.

[2] One of the important results of Fraser's studies is the demonstration that the bile of normal serpents and of certain warm-blooded animals, notably bovines, rabbits, and guinea-pigs, is antitoxic for serpent venom. The antitoxic value of the bile in this connection seems to be fairly comparable to that of other antitoxins obtained from the blood of artificially immunized animals. Its action is not only prophylactic, but also curative, Fraser having succeeded in rescuing animals by the injection of bile-extract thirty minutes after they had received what would otherwise have been a fatal dose of cobra poison. (See Centralblatt für Bakteriologie, 1897, Bd. xxii. S. 420.)

of Behring and Kitasato[1] upon the production of immunity from tetanus. In their studies upon the blood of animals subjected to these experiments it was found that it was not only possible to render animals immune from this disease, but that the serum of the blood of these immunized animals afforded immunity when injected into the peritoneal cavity of other animals that had not been so protected; and moreover, that in some cases this serum possesses curative powers over the disease after it has been in progress for a time. They found, further, that the serum of animals that had been rendered immune from tetanus, when brought in contact with the poison of tetanus, completely destroyed its poisonous properties, and that the serum from animals or from human beings that do not possess immunity from this disease has no such power.

The demonstration by Behring and Kitasato of the fact that the serum of an immunized animal can not only confer immunity upon another susceptible animal, but in the case of tetanus (and diphtheria, as subsequently demonstrated by Behring and his associates), cure the disease after it is already in progress, is one of the most important steps that has been made in this entire field of study. The application of the principle involved in this observation to the cure of diphtheria in man has resulted in a triumph which marks an epoch in modern scientific medicine. The same principle has been employed for obtaining curative agents against other forms of infection and intoxication, notably, of Asiatic cholera, typhoid fever, lobar pneumonia, streptococcus and staphylococcus infection, rabies, tuberculosis,

[1] Behring und Kitasato: Deutsche med. Wochenschrift, 1890, Bd. xvi. S. 1113.

bubonic plague, syphilis, vaccinia, and serpent-venom; but unfortunately, as yet, with only indifferent success; certainly in no case to the same favorable degree as has been seen in the treatment of diphtheria with antitoxic serum.

Another hypothesis in explanation of the immunity acquired by the tissues of the animal organism is that advanced by Buchner,[1] who suggests that in the primary infection, from which the animal has recovered, there has been produced a *reactive change* in the integral cells of the body that enables them to protect themselves against subsequent inroads of the same organism. Though somewhat more vague at first glance than the other theories in regard to this phenomenon, it has, nevertheless, much to recommend it, and in the light of subsequent research is regarded by many as probably the correct explanation of the establishment of immunity in a number of, if not in all, cases. Experiments that bear directly upon this idea have demonstrated that, if animals be subjected to injections of the poisonous products of growth of certain virulent bacteria, they respond to this treatment by more or less pronounced constitutional reactions, and that during this period, and for a short time following, they are protected from the invasion of the virulent bacteria themselves. This observation has, moreover, not been confined to those cases in which injections of the products of growth were followed by inoculations with the bacteria by which they were produced, but, what is still more interesting and confirmatory of Buchner's view, it is claimed that a sort of protection from certain specific

[1] Buchner: Eine neue Theorie uber Erzielung von Immunität gegen Infektionskrankheiten. Muenchen, 1883.

infections can also be afforded to animals by the injection into them of cultures of entirely different species of bacteria, or their products, and that in some cases these are not of necessity of the disease-producing variety. For instance, Emmerich and Mattei[1] claim to have rendered rabbits insusceptible to anthrax through injections into them of cultures of the streptococcus of erysipelas.

This, they claim, is not due to any antagonism between the two organisms themselves, for in culture experiments they grew well together, without any alteration in their pathogenic properties; but rather to the induction of a tissue activity by which resistance to the inroads of the virulent bacilli was established. Emmerich and Mattei interpret this "reactive tissue-change" as a power acquired by the integral cells of the body, through the influence of a stimulus, of generating a product that is detrimental to the pathogenic activity of the anthrax bacilli.

Pawlowsky,[2] who obtained similar results from the introduction into animals of cultures of *bacillus prodigiosus*, of *staphylococcus pyogenes aureus*, and of *micrococcus lanceolatus*, believes them to be due to the induction of increased energy on the part of the wandering cells, preparing them thus for the difficult task of destroying the more virulent organisms with which the animal is subsequently to be inoculated.

Protection afforded in this way apparently contraindicates a specific relation between the morbific elements of particular infections and the protective substances that are present in the body of the animal that

[1] Emmerich und Mattei: Fortschritte der Medicin, 1887, S. 653.
[2] Pawlowsky: Virchow's Archiv, vol. cviii. p. 494.

has been rendered insusceptible to them. It is probable, however, that this is only apparent, and that the observations of Emmerich and Mattei and of Pawlowsky can be interpreted in another way: in the blood of animals there is present what may be termed a normal protective (Buchner's alexins) having no specific relations to any particular variety of infection, but serving to protect the animal more or less completely against all bacterial invasion. By the methods employed in the preceding experiments it seems likely, in the light of more recent work, that this normal antidote was simply *temporarily accentuated* through the tissue-stimulation resultant upon the treatment that the animals had received, for it is not possible to bring about in this way as high or as permanent a degree of immunity in an animal from a particular disease as that which can be obtained by the use of the specific micro-organism causing the disease, or the products of its growth, especially the latter.

A striking illustration of this protective reaction on the part of the animal tissues is brought out in the course of R. Pfeiffer's[1] experiments on Asiatic cholera. He found that it was possible to confer immunity upon animals from this disease; that the blood-serum of such animals protected susceptible animals into which it was injected against what would otherwise be a fatal dose of the cholera spirillum; that the peritoneal fluids of the artificially immunized animal had an almost instantaneous *disintegrating* (bacteriolytic), bactericidal action upon living cholera spirilla that were injected into the peritoneal cavity; that the serum from the immune

[1] Pfeiffer: Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xviii. S. 1; Bd. xx. S. 198

animal had no such effect upon cholera spirilla in the test-tube; but when virulent cholera spirilla were injected into the peritoneum of an animal that was *not* immune, and this was at once followed by an intraperitoneal injection of serum from an *immune* animal, almost instantly the peculiar disintegration of the bacteria, as observed in the peritoneum of the immune animal, could be detected. This latter observation is of the utmost importance in its bearing on Buchner's hypothesis, for we see here a serum from an immune animal that is capable of conferring immunity; capable, on injection into a susceptible animal, of endowing its fluids with the peculiar disintegrating, germicidal function noted in the peritoneum of the immune animal from which the serum originated; incapable of bacteriolytic activity *outside the body*, but the influence of which *in the peritoneum* of a susceptible animal is to call forth at once this interesting phenomenon. Manifestly the germicidal substance in this case is neither contained in the protective serum nor in the peritoneum of the susceptible animal before receiving the serum, but is generated by the tissues as a result of the specific irritation of a something contained in this serum; in other words, in consequence of a reaction on the part of the peritoneal tissues, or possibly those of the entire animal.

In more recent investigations Pfeiffer, in association with Marx, has found that the antitoxic substances in cholera-immune animals are much more abundant in the blood-building organs—spleen, lymphatic glands, and bone-marrow—than in either the blood or other tissues.[1]

[1] Pfeiffer and Marx: Deutsche med. Wochenschrift, 1898, No. 3.

The experiments of G. and F. Klemperer[1] upon acute fibrinous pneumonia, though too limited in extent to be accepted as conclusive, presented nevertheless a number of most significant suggestions, not only in connection with several obscure features of this disease, but in their broader bearing upon acquired tissue-resistance in general.

These authors found but little difficulty in conferring immunity upon animals that are otherwise susceptible to the pathogenic action of the organisms concerned in the production of this disease,[2] by the introduction into their tissues of the products of growth of the organisms from which the latter had been separated. The immunity thus produced is seen in some cases to last as long as six months; again it is seen to disappear suddenly in a way not to be explained. It was seen in one case to be hereditary, probably having been conferred upon the young, during the nursing-period, through the milk of the mother, as Ehrlich[3] has shown to occur in animals artificially immunized from abrin, ricin, and robin.

The energy of the substance that has the power of affording immunity was seen to be very much increased by subjecting it to temperatures somewhat higher than that at which it was produced by the bacteria. The Klemperers found that if this substance was heated to

[1] G. and F. Klemperer: Berliner klin. Wochenschrift, 1891, Nos. 34 and 35.

[2] Animals do not, as a rule, present the pneumonic changes seen in human beings. The introduction of *micrococcus lanceolatus* into their tissues results, in the case of susceptible animals, in the production of septicæmia.

[3] Ehrlich: Zeitschrift für Hygiene und Infektionskrankheiten, 1892, Bd. xii. S. 183.

a temperature of from 41° to 42° C. for three or four days, or to 60° C. for from one to two hours, its intravenous injection was followed by complete immunity in from three to four days; whereas, if the unwarmed material was used, immunity did not appear before fourteen days, and then only after the employment of relatively large amounts. Moreover, when the previously heated products are introduced into the circulation of the animal the systemic reaction is of but short duration; but if the unwarmed substance is employed, immunity is manifest only after the onset of considerable elevation of temperature, which lasts for a long time.

In explanation of these differences they suggest that, in the latter case, the high fever that is seen to occur in the animal may serve to replace the warming to which the bacterial products had not previously been subjected, and which is necessary before they are in a position to bring about the condition of immunity. They claim that the bacterial products employed to produce immunity in this case are not, in reality, the immunity-affording substance, but that they are only the agents that bring about in the tissues of the animal alterations that result in the production of *another body* that protects the animal. In support of this, their argument is that several days are necessary for the production of immunity by the introduction into the animal of the bacterial products; whereas, if the blood-serum of this animal, which is now protected, be introduced into the circulation of another animal, no such delay is seen, but instead the animal is forthwith protected. In the former case the actual protecting body had first to be manufactured by the tissues; whereas

in the second it is already prepared, and is introduced as such into the second animal.

These authors found the serum of artificially immunized animals to be not only capable of rendering other animals immune, but to be possessed of curative powers when the disease was already in progress. The serum of immunized animals when injected into the circulation of animals in which there was a body-temperature of from 40.4° to 41° C. reduced this temperature to normal (37.5° C.) in twelve consecutive experiments during the first twenty-four hours following its employment.

In their opinion, the crisis seen in pneumonia in human beings indicates the moment at which the poisonous products, manufactured by the bacteria located in the lungs, are present in the circulation in amounts sufficient to stimulate the tissues to the reaction that results in the production of the *antidotal* substance that has the power of rendering the poisons inert.

At the time of the crisis in pneumonia the bacteria themselves are in no way affected. They remain in the lungs, and can be detected, in full vigor and virulence, in the sputum of patients a long time after the disease is cured. They have lost none of their power of producing poisonous products, and still possess their original pathogenic relations toward susceptible animals. It is only after the crisis that their poisons are neutralized by this antidotal proteid that has been produced by the cells of the tissues, and as this occurs the systemic manifestations gradually disappear. The Klemperers claim to have isolated from cultures of *micrococcus lanceolatus* a proteid body that is the agent concerned in producing the tissue-reaction which results in

the formation of the protecting substance. They likewise isolated from the serum of immunized animals a proteid that possessed the same powers as the serum itself, viz., of affording immunity and curing the disease.

Here, again, it appears that the processes of infection and immunity are chemical in their nature, the active poisons of the invading organisms—"the pneumotoxins"—being instrumental in producing the diseased condition, while the antidotal or resisting body of the tissues—"the anti-pneumotoxin"—is the agent by which the poison is neutralized.

Results in general analogous to those of G. and F. Klemperer have also been obtained by Emmerich and Fowitzky.[1]

In the light of these experiments the hypothesis advanced by Buchner, that the establishment of immunity is to be explained by reactive changes in the integral cells of the body, receives additional support, and when we consider the observations of Bitter,[2] who found that in protective vaccinations against anthrax the vaccines do not disseminate themselves through the body, as is the case when the virulent organisms are introduced, but remain at the site of inoculation, and from this point produce, by the absorption of their chemical products, the systemic changes through which the animal is protected against subsequent infection by the virulent organisms, we feel justified in concluding that the weight of evidence is strongly in favor of this view.

[1] Emmerich and Fowitzky: Münchener med. Wochenschrift, 1891, No. 32.

[2] Bitter: Zeitschrift für Hygiene, 1887, Bd. iv.

The most recent interpretation of this phenomenon is that proposed by Ehrlich in 1897.[1] It is generally known as his "side chain" or "lateral bond" (Seitenkette) theory of immunity. It is one of the most attractive of all the hypotheses that have been advanced in explanation of this phenomenon, and is in many ways the most satisfactory.

Its important features comprise the acceptance of Weigert's doctrine concerning the *modus operandi* of physiological tissue-equilibrium and repair; and also the assumption of a specific combining relation, or affinity, between toxic substances and the cells of particular tissues.

At the meeting of German Naturalists and Physicians held at Frankfort-on-the-Main, in 1896, Weigert[2] advanced an hypothesis the essential features of which are that physiological structure and function depend upon the equilibrium of the tissues maintained by virtue of mutual restraint between its component cells; that destruction of a single integer or group of integers of a tissue or a cell removes a corresponding amount of restraint at the point injured, and therefore destroys equilibrium and permits of the abnormal exhibition of bioplastic energies on the part of the remaining uninjured components, which activity may be viewed as a compensating hyperplasia; that hyperplasia is not therefore the *direct* result of external irritation, and cannot be, since the destructive action of the irritant is confined to the cells or integers of cells that it destroys, but occurs rather

[1] Ehrlich: Klinisches Jahrbuch, 1897, Bd. vi. Heft 2, S. 309.

[2] Weigert, Carl: "Neue Fragestellungen in der pathologischen Anatomie," Verhandlungen der Ges. deutscher Naturforscher und Aerzte, 1896, S. 121.

indirectly as a function of the surrounding uninjured tissues that have been excited to bioplastic activity through the removal of restraint that had hitherto been exerted by the cells destroyed by the irritant; and, finally, when such bioplastic activity is called into play there is always *hyper*compensation—*i. e.*, there is more plastic material generated than is necessary to compensate for the loss. Ehrlich applies this idea to the individual cell, which he conceives to be a complex molecule, comprising a primary central nucleus to which are attached by side chains its secondary atom-groups having various physiological functions, in much the same way that our conception of the reaction-structure of complex organic chemical compounds is represented graphically. Injury to one or more of these physiologically essential atom-groups results, according to the view of Weigert, in disturbance of the cell-equilibrium and consequent effort on the part of the surrounding atom-groups at compensatory repair. With this liberation of bioplastic energy there is more plastic material generated than is necessary for the repair of the injury. The excess of this material finds its way into the blood and, as we shall presently see, is regarded by Ehrlich as the real antitoxic substance. Assuming a specific combining relation between toxic substances and particular cells or secondary atom-groups of cells—and there are now some experimental grounds for this assumption [1]—it is evident that the combination between the intoxicant and the particular atom-group for which it has a specific affinity is indirectly the cause of com-

[1] See Wassermann und Takaki: "Ueber tetanus antitoxische Eigenschaften des normalen Centralnervensystems," Berliner klin. Wochenschrift, 1898, No. 1, S. 5.

pensatory bioplastic activity on the part of similar surrounding atom-groups that have not been destroyed through combining with the poison. This results, as we learned above, in hypercompensation, the excess of plastic material being disengaged from the parent-cell and thrown free into the circulating fluids, there to combine directly with the same intoxicant should it subsequently gain access to the animal. This excess of plastic material thrown into the circulation combines, according to Ehrlich,[1] directly with the intoxicant to form physiologically inactive toxin—antitoxin compounds (see page 497), and can therefore be reasonably regarded as the *antitoxic* material of artificially immune animals. That portion of the cell which has the specific function of neutralizing, by combining with, the intoxicant Ehrlich designates as the "toxophoric side chain."

Numerous observations indicate the probability of there being present in the blood of animals several bodies having totally different relations to bacteria and their products, according to the conditions under which they exist. First there is present in the blood-serum of practically all animals the normal defensive "alexins" already mentioned. Second, the antitoxins that are found in the blood of animals artificially immunized from special sorts of infection and intoxication, as well as occasionally in the blood and tissues of normal animals, the functions of which are susceptible of demonstration outside the body as well as within the tissues of the living animal. Third, a body possessed of disintegrating, bacteriolytic powers—*i. e.*, having the property of

[1] Ehrlich: "Zur Kenntniss der Antitoxinwirkung," Fortschritte der Medicin, 1897, Bd. xv. No. 2.

actually breaking bacteria to pieces, so that the phenomenon may be observed under the microscope. This phenomenon is especially to be seen within the peritoneum of guinea-pigs that have been rendered immune from Asiatic cholera and from the typhoid and colon infections or intoxications.[1] It is rarely or never seen outside the body, and it is not to be confounded with the ordinary bactericidal function of the alexins that is demonstrable in most normal serums. Fourth, a body, the so-called "agglutinin" (Gruber), that was considered by Widal to represent a "reaction of infection," and not of immunity; though at this time its presence is generally supposed to indicate an effort on the part of the body to resist infection. The presence of this body in a serum is announced by its peculiar influence on the activity and arrangement of particular bacteria with which it is brought in contact. In the case of typhoid fever in man, for instance, the serum obtained during the early and middle stages of the disease, when mixed with fluid cultures or suspensions of the typhoid bacillus, causes the bacilli to lose their motility and to congregate (agglutinate) in masses and clumps, a condition never seen in normal cultures of this organism, and practically never observed when normal serum is employed. There are evidences of the presence of "agglutinin" in certain of the antitoxic serums from artificially immunized animals, viz., that of animals immune from cholera, pyocyaneus, typhoid, and colon infections. So far as experience has gone, this agglutinating property is manifested in the great majority of cases only upon the particular organisms from which the animal is protected; that is to say, the relation is specific.

[1] It is generally known as Pfeiffer's phenomenon.

In view of the fact that its presence has been regarded as indicative of a reaction of infection, its detection in the blood of immune animals may at first sight appear paradoxical. We should not lose sight of the fact, however, that it is assumed to be totally distinct from the other substances concerned in immunity, and its presence in immune animals may, therefore, be reasonably explained as a more or less permanent result of the "reactions of infection" that were coincident with the primary injections into the animal of infective or intoxicating matters necessary to the establishment of the condition of immunity; nor should we in this connection lose sight of the fact that its presence is constantly to be demonstrated in typical cases of typhoid fever, for instance, that terminate fatally, and that have exhibited little or no clinical signs of resistance at any time during their course.

The experiments that have been cited afford but an imperfect idea of the enormous amount of work that has been done upon the manifold phases of these important subjects; they may, however, serve to indicate the direction in which the lines of research have been laid. As a result of such investigations, our knowledge upon infection and immunity may at present be summarized as follows:

1. That infection may be considered as a contest between bacteria and living tissues, conducted on the part of the former by means of the poisonous products of their growth, and resisted by the latter through the agency of proteid bodies normally present in [illegible] erated by their integral cells.

2. That when infection occurs it m[illegible]

either by the excess of vigor of the bacterial products over the antidotal or protective proteids produced by the tissues, or to some cause that has interfered with the normal activity and production of these bodies.

3. That in the serum of the normal circulating blood of many animals there exists a substance that is capable, outside of the body, of rendering inert certain bacteria which, if introduced into the body of the animal, would prove infective.

4. That immunity is most frequently seen to follow the introduction into the body of the products of growth of bacteria that in some way or other have been modified. This modification may be artificially produced in the products themselves of virulent organisms, and then introduced into the tissues of the animal; or the virulent bacteria may be so treated that they are no longer of full virulence, and when introduced into the body of the animal will produce poisons of a much less vigorous nature than would otherwise be the case.

5. That immunity following the introduction of bacterial products into the tissues is not in all cases the result of the continuous presence of these substances, *per se*, in the tissues, or of a tolerance acquired by the tissues to them; but is probably, in certain instances, due to the formation in the tissues of another body or other bodies that act as protecting antidotes to the poisonous products of invading organisms.

6. That this protecting proteid which is generated by the cells of the tissues need not of necessity be antagonistic to the life of the invading organisms themselves, but in some cases must be looked upon more as an antidote to their poisonous products.

7. The immunity, as conceived by Ehrlich, may be either "active" or "passive." According to this interpretation, it is "active" when resulting from an ordinary non-fatal attack of infectious disease; or from a mild attack of infection purposely induced through the use of living vaccines; or from the gradual introduction of toxins into the tissues until a marked stage of tolerance is reached. It is "passive" when occurring as a result of the direct transference of the perfected immunizing substance from an immune to a susceptible animal, as by the injection of blood-serum from the former into the latter. "Passive immunity" is, in most cases, conferred at once, without the delay incidental to the usual modes of establishing "active immunity." As a rule, "active" is more lasting than "passive" immunity.

8. That phagocytosis, though frequently observed, is not essential to the establishment of immunity, but is more probably a secondary process, the bacteria being taken up by the leucocytes only after having been modified in virulence through the normal germicidal activity of the serum of the blood and of other fluids in the body. It is, however, probable that the living leucocytes secrete and add to the circulating fluids certain substances that are important to the establishment of immunity.

9. That of the hypotheses advanced in explanation of acquired immunity, the one worthy of greatest confidence is that which assumes immunity to be due to reactive changes on the part of the tissues that result in the formation in these tissues of antitoxic substances capable of neutralizing the poisons produced by the bacteria against which the animal has been immunized;

though in certain cases the establishment of immunity is accompanied by the assumption of *germicidal* as well as antitoxic properties by the fluids and tissues, and in a few instances the germicidal is more pronounced than the antitoxic function.

CHAPTER XXVII.

Bacteriological study of water—Methods employed—Precautions to be observed—Apparatus used, and methods of using them—Methods of investigating air and soil.

BACTERIOLOGICAL STUDY OF WATER.

THE conditions that favor the epidemic outbreak of typhoid fever, Asiatic cholera, and other maladies of which these may be taken as types, have served as a subject for discussion by sanitarians for a long time.

Of the hypotheses that have been advanced in explanation of the existence and dissemination of these diseases, two stand pre-eminent and are worthy of consideration. They are the "ground-water" theory of von Pettenkofer and his pupils, and the "drinking-water" theory of the school of bacteriologists of which Koch stands at the head.

The adherents to the "ground-water" view explain the occurrence of these diseases in epidemic form through alterations in the soil resulting from fluctuations in the level of the soil-water; and assign to drinking-water either a very insignificant *rôle*, or, as is most frequently the case, ignore it entirely. On the other hand, those who have been instrumental in developing the drinking-water hypothesis claim that alterations in the soil play little or no part in favoring the outbreak of these diseases; but that, as a rule, they appear as a result of direct infection, through the use of waters contaminated

with materials containing the specific organisms known to cause such diseases.

As a result of numerous observations by the disciples of both schools, the evidence is now greatly in favor of the opinion that polluted water is primarily the underlying cause of these epidemics, and this too, very often, when the state of the soil-water, in the light of the "ground-water" hypothesis, is just the reverse of what it should be in order to render it answerable for them. It is manifest, therefore, that the careful bacteriological study of water intended for domestic use is of the greatest importance, and should be a routine procedure in all communities receiving their water-supply from sources liable to pollution.

The object aimed at in such investigations should be to determine the number and kind of bacteria constantly present in the water—for all waters, except deep ground-water, contain bacteria; if sudden fluctuations in the number and kind of bacteria occur in these waters, and if so, to what they are due; and finally, and most important, Does the water contain constantly, or at irregular periods, bacteria that can be traced to human excrement, not of necessity pathogenic varieties, but bacteria that are known to be present normally in the intestinal canal? For if conditions are continuously favorable to pollution of the water by the normal constituents of the intestinal canal, the same conditions would allow of the occasional pollution of such water by infective matters from the *bowels* of persons suffering from specific disease of the intestines.

In considering water from a bacteriological standpoint it must always be borne in mind that comparisons with fixed standards are not of much value,

for just as normal waters from different sources are seen to present variations in their chemical composition, without being unfit for use, so may the relative number and variety of species of bacteria in water from one source be always greater or smaller than in that from another, and yet no difference may be seen to result from their employment. For this reason systematic study of any water, from this point of view, should begin with the establishment of what may be called its normal mean number of bacteria, as well as the character of the prevailing species; and in order to do this the investigations must cover a long period of time through all the seasonal variations of weather. From data obtained in this way it may be possible without analysis to predict approximately at any season the bacteriological condition of the water studied. Marked deviations from these "means," either in the quantity or quality of the organisms present, can then be considered as indicative of the existence of some unusual, disturbing element, the nature of which should be investigated. It is impossible to formulate an opinion of much value from either a single chemical or bacteriological analysis of a water, or from both together in many cases; for the results thus obtained indicate only the condition of the water at the time the sample was procured, and give no indication as to whether it differed at that time from its usual condition, or from the normal condition of the waters of the immediate neighborhood.

The interpretation of the results of both chemical and bacteriological analyses of a sample of water acquires its full value only through comparison, either with "means" that have been determined for this

water, or with the results of simultaneous analyses of a number of samples from other sources of supply of the locality.

The aid of the bacteriologist is frequently sought in connection with investigations of waters that are supposed to be concerned in the production of disease, particularly typhoid fever, either in isolated cases or in widespread epidemic outbreaks, and in these cases both the bacteriologist and the person employing his services are cautioned against being too sanguine of positive results, for in the vast majority of instances reliable bacteriologists fail to detect in these waters the bacillus that is the cause of typhoid fever.

Failure to find the organism of typhoid fever in water by the usual methods of analysis does not by any means prove that it is not present or has not been present. The means ordinarily employed in the work admit of such a very small volume of water being used in the test that we can readily understand how typhoid bacilli might be present in moderate numbers and yet none be included in the drop or two of the water taken for study. The conditions are not those of a *solution*, each drop of which contains exactly as much of the dissolved material as do all other drops of equal volume; but are rather those of a *suspension*, in every drop or volume of which the number of *suspended* particles is liable to the greatest degree of variation. Furthermore, there are other reasons that would, *a priori*, preclude our expecting to find the typhoid bacilli in water in which we may have reason to believe they had been deposited, viz., attention is not usually directed to the water until the disease has become conspicuous, usually in from two to four

weeks after the pollution probably occurred. These intervals of time are ordinarily sufficient for the delicate, non-resistant bacillus of typhoid fever to succumb to the unfavorable conditions under which it finds itself in water. By unfavorable conditions are meant the absence of suitable nutrition; unfavorable temperature; probably the antagonistic influence of more hardy saprophytic bacteria, particularly the so-called "water-bacteria," and of more highly organized water-plants; the effect of precipitation and of sedimentation; and, of great importance, the disinfecting action of direct sunlight.

Though the positive demonstration of typhoid bacilli in drinking-water by bacteriological methods is of extreme rarity, it must not be concluded that bacteriological analyses of suspicious waters shed no light upon the existence of pollution and the suitability or non-suitability of the water for drinking-purposes.

In the normal intestinal tract of all human beings and of many other mammals, as well as associated with the specific disease-producing bacterium in the intestines of typhoid-fever patients, is an organism that is frequently found in polluted drinking-waters, and whose presence is proof positive of pollution by either normal or diseased intestinal contents; and though efforts may result in failure to detect the specific bacillus of typhoid fever, the finding of the other organism, *bacterium coli commune*, justifies one in expressing the opinion that the water under consideration has been polluted by intestinal evacuations from either human beings or animals. Waters so exposed as to be liable to such pollution should never be considered as other than a continuous source of danger to those using them.

Another point to be remembered is in connection with chlorine as an indicator of contamination by human excrement. It is commonly taught that an excessive amount of chlorine in water points to contamination by human excreta. This may or may not be true, according to circumstances. A high proportion of this element in a sample of water from a locality, the surrounding waters of which are poor in chlorine, is unquestionably a suspicious indication; but in a district close to the sea or near salt-deposits, for instance, where the proportion of chlorine (as chlorides) in the water is generally high, the value of the indications thus afforded is very much diminished unless the amount found in the sample under examination greatly exceeds the normal "mean," previously determined, for the amount of chlorine in the waters of the neighborhood.

A striking example of the latter condition occurred in the experience of the writer while inspecting a group of water-supplies on the east coast of Florida. In each instance the water was obtained from properly protected artesian wells, ranging from 200 to 400 feet deep, and located within a few hundred yards of the sea. The first sample subjected to chemical analysis revealed such an unusually high proportion of chlorine that, had this sample alone been considered, the opinion that it was polluted by human excreta might have been advanced. To prevent such an error samples of water from a number of wells in the neighborhood were examined, and they were all found to contain from ten to twelve times the amount of chlorine that ordinarily appears in inland waters, the excess being evidently due to leakage through the soil into the wells of water from the sea. In short, the

presence of an excess of chlorine in water, while often indicating pollution from human evacuations, may nevertheless, sometimes arise from other sources; but the presence in water of bacteria normally found in the intestinal canal can manifestly admit of but one interpretation, viz., that fæcal matters from either man or animals have at some time been deposited in this water, and that while no specific disease-producing organisms may have been detected, still waters in which such pollutions are possible are a constant menace to the health of those who use them for domestic purposes.

A sudden variation from the normal, mean number of bacteria, or from the normal chemical composition of a water, calls at once for a thorough inspection of the supply, while at the same time the organisms present are to be subjected to the most careful study. In many instances, even after the most thorough bacteriological and chemical study of a suspicious water, one is forced to admit that information of but limited usefulness has been obtained through the employment of such analytical methods. In these cases too much stress cannot be laid upon the importance of a systematic inspection of the supply, and its relation to sources of pollution. Optical evidence of more or less dangerous contamination may often be obtained when laboratory methods fail to detect them. The reasons for such failure, in addition to those already given, are obvious—the polluting matters are often so diluted by the large mass of water into which they find their way as to be beyond recognition by the tests usually employed in such work, and still be present in amounts sufficient to originate disease.

THE QUALITATIVE BACTERIOLOGICAL ANALYSIS

OF WATER.—The qualitative bacteriological analysis of water entails much labor, as it requires not only that all the different species of organisms found in the water should be isolated, but that each representative should be subjected to systematic study, and its pathogenic or non-pathogenic properties determined.

For this purpose a knowledge of the methods for the isolation of individual species which have already been described, and of the means of studying these species when isolated, is indispensable.

For this analysis certain precautions essential to accuracy are always to be observed.

The sample is to be collected under the most rigid precautions that will exclude organisms from sources other than that under consideration. If drawn from a spigot, it should never be collected until the water has been flowing for fifteen to twenty minutes in a full stream. If obtained from a stream or a spring, it should be collected, not from the surface, but rather from about one foot beneath the surface.

It should always be collected in vessels which have previously been thoroughly freed from all dirt and organic particles, and then sterilized; and the plates should be made as quickly as possible after collecting the sample.

When circumstances permit, all water analyses should be made on the spot where the sample is taken, as it is known that during transportation, unless the samples are kept packed in ice, a multiplication of the organisms contained in it always occurs.

For the purpose of qualitative analysis it is necessary that a small portion of the water—one, two, three, five drops—should first be employed for making the plates.

In this way one can form an idea as to the approximate number of organisms in the water, and can, in consequence, determine the amount of water best suited for the plates. Duplicate plates are always to be made—one set upon agar-agar, which are to be kept in the incubator at body-temperature, and one set upon gelatin, to be kept at from 18° to 20° C.

As soon as colonies have developed the plates are to be carefully compared and studied. It is to be noted if any difference in the appearance of the organisms on corresponding plates exists, and if so, to what it is due. It is to be particularly noted which plates contain the greater number of colonies, those kept at the higher or those at the lower temperature. In this way the temperature best suited for the growth of the majority of these organisms may be determined. As a rule, the greater number of colonies appear upon the gelatin plates kept at 18° to 20° C.; and from this it would seem that many of the normal water-bacteria do not find the higher temperature so favorable to their development as do the organisms not naturally present in water, particularly the pathogenic varieties. From these plates the different species are to be isolated in pure culture, the morphological and cultural characteristics determined, and finally, by tests upon animals, it is to be decided if any of them possess disease-producing properties.

NOTE.—What use should be made of this observation in examining water for the presence of pathogenic bacteria?

The waters most frequently studied from the qualitative bacteriological standpoint are those suspected

of containing specific pathogenic bacteria—*i. e.*, waters polluted with sewage and with human excreta that are believed to be the source of infection of typhoid fever, or, less frequently, of Asiatic cholera. In the investigations of such waters there are several points of which we should never lose sight, viz., unless the water is under continuous study there is only a chance of detecting the specific pathogenic species, for, as a rule, the dangerous pollution occurs either but once or is intermittent, so that even in the case of exposed streams there are periods when no specifically dangerous contamination may be in operation. As stated above, attention is commonly called to the water when the disease, presumably caused by its use, is fully developed, and this is often days or weeks after the infection of the stream really occurred. By an analysis made at this time one could scarcely hope to detect the specific organisms that had caused the disease. The organisms sought for may have been present in the water and may have infected the users, and yet have disappeared by the time the sample taken for analysis was collected.

When present in polluted waters pathogenic bacteria are always vastly in the minority. Were they constantly present in large numbers infection among the users of such waters would be more frequent and more widespread than is commonly the case. They may be present in a water-supply in small numbers; they may even be in the sample supplied for analysis, and yet escape detection if only the ordinary direct plate method of isolation be used.

From these considerations it is obvious that before attempts are made to isolate the various species directly from a suspicious sample of water it is advisable to

subject it to some method of treatment that will aid in separating the few specific pathogenic from the numerous common saprophytic species. For this purpose numerous methods have been devised. The most useful of these aim to favor the rapid multiplication of pathogenic forms that may be present and to suppress or check the growth of the ordinary water saprophytes.

Attention has been called to the fact that when exposed to the body-temperature many of the ordinary water-bacteria develop only slowly or not at all, while under similar circumstances the disease-producing species develop most luxuriantly. Advantage has been taken of this observation in devising methods for this particular work, of which some of the following will prove serviceable:

Collect in a sterilized flask a sample of about 100 c.c. of the water to be tested, and add to this about 25 c.c. of sterilized bouillon of *four times the usual strength*. This is then placed in the incubator at 37° to 38° C., for thirty-six to forty-eight hours, after which plates are to be made from it in the usual way; the results will often be a pure culture of some single organism, either one of the intestinal variety or a closely allied species. By a method analogous to the latter the spirillum of Asiatic cholera has been isolated from water (see pages 110, 120); and by taking advantage of the effect of elevated temperature upon the bacteria of water Vaughan has succeeded in isolating from suspicious waters a group of organisms very closely allied to the bacillus of typhoid fever.

Theobald Smith has suggested a method by which it is easily possible to isolate, from waters in which they are present, certain organisms that are of the utmost

importance in influencing our judgment upon the fitness of the water for domestic use. By the addition of small quantities—one, two, or three drops—of the suspicious water to fermentation-tubes (see article an Fermentation-tube) containing bouillon to which 2 per cent. of glucose has been added, and keeping them at the temperature of the body (37° to 38° C.), the growth of intestinal bacteria that may be present in the water is favored, while that of the water-organisms is not; in consequence, after from thirty-six to forty-eight hours the fermentation-characteristics of most of these organisms is evidenced by the accumulation of gas in the closed end of the tube. From these tubes the growing bacteria can then be easily isolated by the plate method, and intestinal bacteria will not infrequently be found present.

For the isolation of the typhoid bacillus, especially from water, a host of other methods have been devised. Some of these aim, through the addition of special chemical reagents to the media, to retard the development of ordinary saprophytes without interrupting the growth of the colon and the typhoid bacillus. Most of these methods have proved disappointing. One of them, that of Parietti, still finds favor in the hands of some. It consists in adding to the culture-media to be used in the test varying amounts of the following mixture:

Phenol	5 grammes.
Hydrochloric acid	4 "
Distilled water	100 c.c.

Of this solution 0.1, 0.2, and 0.3 c.c. are added respectively to each of three tubes containing 10 c.c. of nutritive bouillon. Several such sets of tubes are to be

made. To each are then added from 1 to 3 c.c. of the water, and they are placed in the incubator at body-temperature. It is said that whatever development occurs consists only of the typhoid or colon bacillus, or both, if they were present in the original sample. They may then be isolated and separated by the usual plate method, or, better still, through the application of the methods of Elsner, Stoddart, or Hiss, detailed in the chapter on *bacillus typhosus*. Personally we have not had much success with the Parietti method. The typhoid bacillus has been isolated from water by passing very large quantities of water through an ordinary Pasteur or Birkfield filter, brushing off the matters collected on the filter into a sterilized vessel and examining this by plate methods.

It has occurred to us that possibly the employment of chemical coagulants, such as alum and iron, might prove serviceable for this purpose. Their action would be to mechanically drag down, in precipitating as hydroxides, the suspended bacteria contained in the fluid. This precipitate could then be examined bacteriologically, instead of the water.

The difficulties in this field of work are obviously due to the suspension of a very small number of the disease-producing organisms sought for in large volumes of fluid, and the association with them of large numbers of other species that offer a very great obstacle to the successful search for the pathogenic varieties.

If by either of the above procedures bacilli that bear any resemblance to *bacillus typhosus* be isolated, recourse must then be had to all the differential tests detailed in the chapter on that organism.

THE QUANTITATIVE ESTIMATION OF BACTERIA IN

WATER.—Quantitative analysis requires more care in the measurement of the exact volume of water employed, for the results are to be expressed in terms of the number of individual organisms to a definite volume. The necessity for making the plates at the place at which the sample is collected is to be particularly accentuated in this analysis, for multiplication of the organisms during transit is so great that the results of analyses made after the water has been in a vessel for a day or two are often very different from those that would have been obtained on the spot.

NOTE.—Inoculate a tube containing about ten cubic centimetres of sterilized distilled or tap water with a very small quantity of a solid culture of some one of the organisms with which you have been working, taking care that none of the culture-medium is introduced into the water-tube and that the bacteria are evenly distributed through it. Make plates at once, and on each succeeding day, from this tube, and determine by counts whether there is an increase or diminution in the number of organisms—*i. e.*, if they are growing or dying. Represent the results graphically, and it will be noticed that in many cases there is during the first three or four days a multiplication, after which there is a rapid diminution; and, if the organism does not form spores, usually death in from ten to twelve days. This is not true for all organisms, but does hold for many.

Where it is not convenient, however, to make the analysis on the spot, the sample of water should be taken and packed in ice and kept on ice until the plates

can be made from it, which should in all cases be as soon after its collection as possible.

For the collection of water for this purpose a convenient vessel is a glass bulb (Fig. 100) or balloon, which is easily blown from glass tubing.

FIG. 100.

Glass bulb for collecting samples of water.

It consists simply of a round glass sphere blown on the end of a glass tube, which latter is subsequently drawn out into a capillary stem and sealed while hot. As it cools, the contraction of the air within the bulb results in the establishment of a negative pressure.

If the point of the stem be broken off under water, the water flows into the bulb, because of the existence of the negative pressure within. The negative pressure obtained in this way is frequently insufficient for filling the bulb, and often only a few drops of fluid can be obtained. To obviate this, bulbs may be blown and allowed to cool, but not sealed. After a sufficient number of them are prepared they are taken, one at a time, and gently warmed over a flame; while still warm the extremity of the stem is dipped into distilled water and held there until a few drops have passed up into the bulb; this is then carefully boiled, or, rather, *completely vaporized*, over the flame, and while steam is still escaping the point is sealed in the gas-flame. All air will thus have been replaced by water-vapor, and if the point of the stem be now broken off under water

the bulb will fill quickly. It is not desirable to fill them *completely*, but rather to only about three-fourths of their capacity, as when *full* it is difficult to empty them without contaminating the contents. They are emptied by *gently* warming over a gas or alcohol flame.

A number of bulbs may be made, sealed, and kept on hand. They are sterile so long as they are sealed, because of the heat employed in their manufacture.

When a sample of water is to be taken, the point of a bulb is simply broken off with sterilized forceps under water and held there until the necessary amount has been obtained. This may serve as a sample from which to prepare plates or Esmarch tubes on the spot; or the tip of the stem may be resealed in the flame of an alcohol lamp, the bulb packed in ice, and transported in this condition to the laboratory.

Another very simple and useful device for collecting water samples is that recommended by Kirschner. This consists of a piece of glass tubing of about 5 or 6 mm. inside diameter, and 36 cm. long, bent in the form of a U, with either extremity of the arms bent again at right angles in the same plane and drawn out to a point and sealed. Such tubes are sterilized in the flame as they are made. The sample is collected by breaking off both points, immersing one of them in water and sucking on the other until the tube is filled. Then both points are again sealed in the flame and the tube packed in ice. The objection to this tube is the danger of contaminating its contents with saliva during the act of filling by suction, though this danger is not so great as might at first appear, as we shall learn in our efforts to cultivate bacteria from the mouth-cavity.

NOTE.—Make cover-slips from your own mouth; make plates on both gelatin and agar-agar, at the same time. Compare the number of bacteria found by microscopic examination of the cover-slips with the number of colonies that develop on the plates.

FIG. 101.

Bottle for collecting water.

For the collection of samples from the deeper portions of streams, lakes, etc., a number of convenient devices have been made. A very satisfactory apparatus has been made for me by Messrs. Charles Lentz & Sons, of Philadelphia. It consists of a metal framework, in which is encased a bottle provided with a ground-glass stopper. To the stopper a spring clamp is attached, and this in turn is operated by a string, so that when the weighted apparatus is allowed to sink into the stream the stopper may be removed from the bottle at any depth by simply pulling upon the string. When the bottle is filled with water the stopper is allowed to spring back into position by releasing the string. The whole apparatus (depicted in Fig. 101) is provided with a weight that insures its sinking, and a heavy cord by which it may be lowered and raised. It should be sterilized before using. After collecting the sample the bottle should be wiped dry with a sterilized towel. Before removing the stopper the mouth of the bottle should be rinsed with alcohol

and heated with a gas-flame, to prevent contamination of its contents by matters that may have been upon its surface.

In beginning the quantitative analysis of water with which one is not acquainted certain preliminary steps are essential.

It is necessary to know approximately the number of organisms contained in any fixed volume, so as to determine the quantity of water to be employed for the plates or tubes. This is usually done by making preliminary plates from one drop, two drops, 0.25 c.c., 0.5 c.c., and 1 c.c. of the water. After each plate has been labelled with the amount of water used in making it, it is placed aside for development. When this has occurred one selects the plate upon which the colonies are only moderate in number—about 200 to 300 colonies presenting —and employs in the subsequent analysis the same amount of water that was used in making this plate.

If the original water contained so many organisms that there developed on a plate or tube made with one drop too many colonies to be easily counted, then the sample must be diluted with one, ten, twenty-five, fifty, or one hundred volumes, as the case may require, of sterilized distilled water. This dilution must be *accurate*, and its exact extent noted, so that subsequently the number of organisms per volume in the original water may be calculated.

The use of a drop is not sufficiently accurate. The dilution should therefore always be to a degree that will admit of the employment of a volume of water that may be exactly measured, 0.25 and 0.5 c.c. being the amounts most convenient for use.

Duplicate plates should always be made, and the

mean of the number of colonies that develop upon them taken as the basis from which to calculate the number of organisms per volume in the original water.

For example: from a sample of water 0.25 c.c. is added to a tube of liquefied gelatin, carefully mixed and poured as a plate. When development occurs the number of colonies are too numerous to be accurately counted. One cubic centimetre of the original water is then to have added to it, under precautions that prevent contamination from without, 99 c.c. of sterilized distilled water—that is, we have now a dilution of 1 : 100. Again, 0.25 c.c. of this dilution is plated, and we find 180 colonies on the plate. Assuming that each colony develops from an individual bacterium, though this is perhaps not strictly true, we had 180 organisms in 0.25 c.c. of our 1 : 100 dilution; therefore in 0.25 c.c. of the original water we had $180 \times 100 = 18{,}000$ bacteria, which will be 72,000 bacteria per cubic centimetre (0.25 c.c. = 18,000, 1 c.c. = $18{,}000 \times 4 = 72{,}000$). The results are always to be expressed in terms of the number of bacteria per cubic centimetre of the original water.

Another point of very great importance (already mentioned) is the effect of temperature upon the number of colonies of bacteria that will develop on the plates made from water. It must always be remembered that a larger number of colonies appear on gelatin plates made from water and kept at 18° to 20° C. than on agar-agar plates kept in the incubator. The following table, illustrative of this point, gives the results of parallel analyses of the same waters, the one series of counts having been made upon gelatin plates at the ordinary temperature of the room, the other upon plates of agar-agar

kept for the same length of time in the incubator at from 37° to 38° C. It will be seen from the table that much the larger number of colonies—*i. e.*, much higher results—were always obtained when gelatin was employed. The importance of this point in the quantitative bacteriological analysis of water is too apparent to require further comment.

TABLE COMPARING THE RESULTS OBTAINED BY THE USE OF GELATIN AT 18°-20° C. AND AGAR-AGAR AT 37°-38° C. IN QUANTITATIVE BACTERIOLOGICAL ANALYSES OF WATER. RESULTS RECORDED ARE THE NUMBER OF COLONIES THAT DEVELOPED FROM THE SAME AMOUNT OF VARIOUS WATERS IN EACH SERIES.[1]

Number of Colonies from Water that developed upon—	
Gelatin plates at 18° to 20° C.	Agar-agar plates at 37° to 38° C.
310	170
280	140
310	180
340	160
650	210
630	320
380	290
400	210
1000	100
890	130
340	280
370	210
490	110
580	100

Another point of equal importance in its influence upon the number of colonies that develop is the reaction of the gelatin. A marked excess of either alkalinity or acidity always has a retarding effect upon many species found in water. Fuller's experience at the Lawrence (Mass.) Ex-

[1] I am indebted to James Homer Wright, Thomas Scott Fellow in Hygiene (1892 '93), University of Pennsylvania, for the results presented in this table.

periment Station has shown that gelatin of such a degree of acidity as to require the *further* addition of from 15 to 20 c.c. per litre of a normal caustic alkali solution to bring it to the phenolphtalein neutral point gives, on the whole, the best results. Thus, by way of illustration, Fuller found that a sample of Merrimac River water gave 5800 colonies per c.c. on phenolphtalein *neutral* gelatin, 15,000 colonies on gelatin that *would need* 20 c.c. of normal alkali solution to bring it up to the phenolphtalein neutral point—*i. e.*, a feebly acid nutrient gelatin, and 500 colonies on a gelatin so alkaline as to require 20 c.c. of a normal *acid* solution to bring it back to the phenolphtalein neutral point.

Throughout this part of the work it is to be borne in mind that when reference is made to plates it is not to a set, as in isolation experiments, but to a single plate.

Method of Counting the Colonies on Plates.—For convenience in counting colonies on plates or in tubes it is customary to divide the whole area of the gelatin occupied by colonies into smaller areas, and either count all the colonies in each of these areas and add the several sums together for the total, or to count the number of colonies in each of several areas, ten or twelve, take the mean of the results and multiply this by the number of areas containing colonies. The latter procedure obtains, of course, only when all the areas are of the same size. By this method, however, the results vary so much in different counts of *the same plate* that they cannot be considered as more than rough approximations.

Note.—Prepare a plate; calculate the number of colonies upon it by this latter method. Now repeat

the calculation, making the average from another set of squares. Now actually count the entire number of colonies on the plate. Compare the results.

For facilitating the counting of colonies several very convenient devices exist.

WOLFFHÜGEL'S COUNTING-APPARATUS.—This apparatus (Fig. 102) consists of a flat wooden stand, the centre of which is cut out in such a way that either a black or white glass plate may be placed in it. These form a background upon which the colonies may more easily be seen when the plate to be counted is placed

FIG. 102.

Wolffhügel's apparatus for counting colonies.

upon it. When the gelatin plate containing the colonies has been placed upon this background of glass it is covered by a transparent glass plate which swings on a hinge. This plate, which is ruled in square centimetres and subdivisions, when in position is just above the colonies, without touching them. The gelatin plate is moved about until it rests under the centre of the area occupied by the ruled lines. The number of colonies

in each square centimetre is then counted, and the sum-total of the colonies in all these areas gives the number of colonies on the plate; or, as has already been indicated, if the number of colonies be very great, a mean may be taken of the number in several (six or eight) squares; this is to be multiplied by the total number of squares occupied by the gelatin. The result is an approximation of the total number of colonies.

When the colonies are quite small, as is frequently the case, the counting may be rendered easier by the use of a small hand-lens. (Fig. 103.)

FIG. 103.

Lens for counting colonies.

Several useful modifications of this apparatus have been introduced. The most important is that of Lafar.[1] Lafar's counter consists of a glass disk of the diameter of ordinary size Petri dishes. It is supplied with a collar or flange that fits around the bottom of the Petri dish, and thus holds the counter in position. The disk is ruled with concentric circles, and its area is divided into sectors of such sizes that the spaces between the concentric circles and the radii forming the sectors are of equal size. Three of the sectors are subdivided into smaller areas of equal size for convenience in counting when the colonies are very numerous. The principles involved

[1] Centralblatt für Bakteriologie und Parasitenkunde, 1891, Bd. xv. S. 331.

are similar to those of the preceding apparatus, but the circular form of the apparatus admits of more exactness when counting colonies on a circular plate.[1]

Pakes[2] has introduced a cheap and convenient modification of Lafar's apparatus. It consists of a sheet of white paper on which is printed a black disk ruled

Fig. 104.

Pakes's apparatus for counting colonies (reduced one-third).

with white lines, in somewhat the same fashion as is Lafar's counter, though the areas of the smallest subdivisions are not of one size and do not bear a constant

[1] Lafar's apparatus is to be obtained from F. Mollenkopf, 10 Thorstrasse, Stuttgart, who holds the patent for it. Its price is about 8 marks.

[2] Journal of Bacteriology and Pathology, 1896, vol. iv. No. 1.

relation to each other.[1] To use this apparatus (Fig. 104) the Petri dish is placed centrally upon it, the cover of the dish is removed, and the colonies are counted as they lie over the spaces bounded by the white lines on the black disk beneath. When the plate is centred over the black disk the portion lying over one sector is exactly one-sixteenth of the whole plate.

ESMARCH'S COUNTER.—Esmarch devised a counter (Fig. 105) for estimating the number of colonies present

FIG. 105.

Esmarch's apparatus for counting colonies in rolled tubes.

upon a cylindrical surface, as when in rolled tubes. The principles and methods of estimation are practically the same as those given for Wolffhügel's apparatus.

A simpler method than by the use of Esmarch's

[1] Copies of this apparatus are to be had of Ash & Co., 42 Southwark Street, London, or of Lentz & Sons, North Eleventh Street, Philadelphia, Pa. (The cost is but a few cents per copy.)

apparatus may be employed for counting the colonies in rolled tubes. It consists in dividing the tube by lines into four or six longitudinal areas, which are subdivided by transverse lines about 1 or 2 cm. apart. The lines may be drawn with pen and ink. They need not be exactly the same distance apart nor exactly straight. Beginning with one of these squares at one end of the tube, which may be marked with a cross, the tube is twisted with the fingers, always in one direction, and the exact number of colonies in each square as it appears in rotation is counted, care being taken not to count a square more than once; the sums are then added together, and the result gives the number of colonies in the tube. This method may be facilitated by the use of a hand-lens.

In all these methods there is one error difficult to eliminate: it is assumed that each colony has grown from a single organism. This is probably not always the case, as there may exist clumps of bacteria which represent hundreds or even thousands of individuals, but which still give rise to but a single colony—obviously this is of necessity estimated as a single organism in the water under analysis.

Where grounds exist for suspecting the presence of these clumps they may in part be broken up by shaking the original water with sterilized sand.

What has been said for the bacteriological examination of water holds good for all fluids which are to be subjected to this form of analysis.

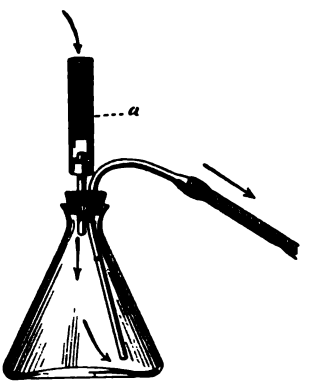
BACTERIOLOGICAL ANALYSIS OF AIR.

Quite a number of methods for the bacteriological study of the air exist. In the main they consist either

of allowing air to pass over solid nutrient media (Koch, Hesse) and observing the colonies which develop upon the media, or of filtering the bacteria from the air by means of porous and liquid substances, and studying the organisms thus obtained. (Miguel, Petri, Strauss, Würz, Sedgwick-Tucker.) Because of their greater exactness, the latter have supplanted the former methods.

In some of the methods which provide for the filtration of bacteria from the air by means of liquid substances a measured volume of air is aspirated through liquefied gelatin; this is then rolled into an Esmarch tube and the number of colonies counted, just as was done in water analysis. This is the simplest procedure. An objection sometimes raised against it is that organisms

FIG. 106.



Petri's apparatus for bacteriological analysis of air. The tube packed with sand is seen at the point *a*.

may be lost, and not come into the calculation, by passing through the medium in the centre of an air-bubble without being arrested by the fluid—an objection that appears to have more of speculative than of real value.

Filtration through porous substances appears, on the whole, to give the best results. Petri recommends aspiration of a measured volume of air through glass tubes into which sterilized sand is packed. (Fig. 106.) When aspiration is finished the sand is mixed with liquefied gelatin, plates are made, and the number of developing colonies counted, the results giving the number of organisms contained in the volume of air aspirated through the sand.

The main objection to this method is the possibility of mistaking a sand-granule for a colony. This objection has been overcome by Sedgwick and Tucker, who employ granulated sugar instead of sand; this, when brought into the liquefied gelatin, dissolves, and no such error as that possible in the Petri method can be made.

Sedgwick-Tucker Method.—On the whole, the method proposed by Sedgwick and Tucker gives such uniform results that it is to be preferred to others. It is as follows:

The apparatus employed by them consists essentially of three parts:

1. A glass tube of special form, to which the name *aërobioscope* has been given.

2. A stout copper cylinder of about sixteen litres capacity, provided with a vacuum-gauge.

3. An air-pump.

The aërobioscope (Fig. 107) is about 35 cm. in its entire length; it is 15 cm. long and 4.5 cm. in diameter at its expanded part; one end of the expanded part is narrowed to a neck 2.5 cm. in diameter and 2.5 cm. long. To the other end is fused a glass tube 15 cm. long and 0.5 cm. inside diameter, in which is to be placed the filtering-material.

Upon this narrow tube, 5 cm. from the lower end, a mark is made with a file, and up to this mark a small roll of brass-wire gauze (*a*) is inserted; this serves as a stop for the filtering-material which is to be placed over it. Beneath the gauze (at *b*), and also at the large end (*c*), the apparatus is plugged with cotton.

FIG. 107.

The Sedgwick-Tucker aërobioscope.

When thoroughly cleaned, dried, and plugged, the apparatus is to be sterilized in the hot-air sterilizer. When cool the cotton plug is removed from the large end (*c*), and thoroughly dried and sterilized No. 50 granulated sugar is poured in until it just fills the 10 cm. (*d*) of the narrow tube above the wire gauze. This column of sugar is the filtering-material employed to engage and retain the bacteria. After pouring in the sugar the cotton-wool plug is replaced, and the tube is again sterilized at 120° C. for several hours.

Taking the air sample. In order to measure the amount of air used the value of each degree on the vacuum-gauge is determined in terms of air by means of an air-meter, or by calculation from the known capacity of the cylinder. This fact ascertained, the negative pressure indicated by the needle on exhausting the cylinder shows the volume of air which must pass into it in order to fill the vacuum. By means of the air-

pump one exhausts the cylinder until the needle reaches the mark corresponding to the amount of air required.[1]

A sterilized aërobioscope is now to be fixed in the upright position and its small end connected by a rubber tube with a stopcock on the cylinder, or to a glass tube tightly fixed in the neck of an aspirating-bottle by means of a perforated rubber stopper. The cotton plug is then moved from the upper end of the aërobioscope, and the desired amount of air is aspirated through the sugar. Dust-particles and bacteria will be held back by the sugar. During manipulation the cotton plug is to be protected from contamination.

When the required amount of air has been aspirated through the sugar the cotton plug is replaced, and by gently tapping the aërobioscope while held in an almost horizontal position the sugar, and with it the bacteria, are brought into the large part (*e*) of the apparatus. When all the sugar is thus shaken down into this part of the apparatus about 20 c.c. of liquefied, sterilized gelatin is poured in through the opening at the end *c*, the sugar dissolves, and the whole is then rolled on ice, just as is done in the preparation of an ordinary Esmarch tube.

The gelatin is most easily poured into the aërobioscope by the use of a small, sterilized, cylindrical funnel (Fig. 108), the stem of which is bent to an angle of about 110° with the long axis of the body.

[1] Such a cylinder and air-pump are not necessary. A pair of ordinary aspirating-bottles of known capacity graduated into litres and fractions thereof answer perfectly well. Or one can determine by the weight of water that has flowed from the aspirator the volume of air that has passed in to take its place; *i. e.*, the volume of air that has passed through the aërobioscope.

The larger part of the aërobioscope is divided into squares to facilitate the counting of the colonies.

By the employment of this apparatus one can filter the air at any place, and can then, without fear of contamination, carry the tubes to the laboratory and complete the analysis. Aside from this advantage, the filter

FIG. 108.

Bent funnel for use with aërobioscope.

being soluble only the insoluble bacteria are left imbedded in the gelatin.

For general use this method is to be preferred to the others that have been mentioned.

BACTERIOLOGICAL STUDY OF THE SOIL.

Bacteriological study of the soil may be made by either breaking up small particles of earth in liquefied media and making plates directly from this; or by what is perhaps a better method, as it gets rid of insoluble particles which may give rise to errors: breaking up

the soil in sterilized water and then making plates immediately from the water.

It must be borne in mind that many of the ground-organisms belong to the anaërobic group, so that in these studies this point should be remembered and the methods for the cultivation of such organisms practised in connection with the ordinary methods. It must also be remembered that the nitrifying organisms, everywhere present in the ground, cannot be isolated by the ordinary methods, and will not appear in plates made after either of the above plans. The special devices for their cultivation are described in the chapter on soil-organisms.

CHAPTER XXVIII.

Methods of testing disinfectants and antiseptics—Experiments illustrating the precautions to be taken—Experiments in skin-disinfection.

DETERMINATION OF DISINFECTANT PROPERTIES.

THERE are several ways of determining the germicidal value of chemical substances, the most common being to expose organisms dried upon bits of silk thread to the disinfectant for different lengths of time, and then, after removing, and carefully washing the threads in water, to place them in nutrient media at a favorable temperature, and notice if any growth appears. If no growth results, the disinfection is presumably successful. Another method is to mix fluid cultures of bacteria with the disinfectant in varying proportions, and, after different intervals of time, to determine if disinfection is in progress by transferring a portion of the mixture to nutrient media, just as in the other methods of work.

By the first of these processes the bits of thread, usually about 1 to 2 cm. long, are placed in a dry test-tube provided with a cotton plug and carefully sterilized, either by the dry method or in the steam sterilizer, before using. They are then immersed in a pure bouillon culture or in a salt-solution suspension of the organism upon which the disinfectant is to be tested. I say "pure culture," because it is always desirable in testing a substance to determine its germicidal value

for several different resistant species of bacteria, both in the vegetating and in the spore stage, and also because it is only by the use of pure cultures of *familiar* species that it is possible to distinguish between the colonies growing from the individuals that have not been destroyed by the disinfectant under investigation and those of unknown species that may appear upon the plate as contaminations occurring during the manipulation.

After the threads have remained in the culture or suspension for from five to ten minutes they are removed under antiseptic precautions and carefully separated and spread upon the bottom of a sterilized Petri dish, which is then placed either in the incubator at a temperature not exceeding 38° C. until the excess of fluid has evaporated, or in a desiccator over sulphuric acid, calcium chloride, or any other drying-agent. The threads are not left there until *absolutely* dry, but only until the *excess* of moisture has evaporated. When sufficiently dry they are immersed in solutions of the disinfectant of different but known strengths for a fixed interval of time, say one or two hours, after which they are removed, rinsed in sterilized distilled water to remove the excess of disinfectant adhering to them, and placed in fresh, sterile culture-media, which are then placed in the incubator at from 37° to 38° C. If after twenty-four to forty-eight or seventy-two hours a growth occurs at or about the bit of thread, and if this growth consists of the organism with which the test was made, manifestly there has been no disinfection; if no growth occurs after, at most, ninety-six hours, it is safe to presume that the bacteria have been killed, unless our efforts at rinsing off the excess of disinfectant from

the thread have not been successful, and a small amount of disinfectant is still active in preventing development —*i. e.*, is acting as an antiseptic.

By the process in which cultures or suspensions of the organisms are mixed with different but known strengths of the disinfectant a small portion of the mixture, usually a loopful or a drop, is transferred at the end of a definite time to the fresh medium which is to determine whether the organisms have been killed or not. This is commonly a tube of fluid agar-agar, which is poured into a Petri dish, allowed to solidify, and placed in the incubator, as in the preceding method.

After the *minimum strength* of disinfectant necessary to destroy the vitality of the organisms with which we are working has been determined for any fixed time, it remains for us to decide what is the *shortest time* in which this strength will have the same effect. We then work with a constant dilution of the disinfectant, but with varying intervals of exposure—one, five, ten minutes, etc.—until we have decided not only the minimum amount of disinfectant required for the destruction of the bacteria, but the shortest time necessary for this under known conditions.

A factor not to be lost sight of is the temperature at which these experiments are conducted, for it must always be borne in mind that the action of a disinfectant is usually *more energetic* at a *higher* than at a lower temperature.

Now in both of these methods it is easy to see that unless special precautions are taken a minute portion of the disinfectant may be carried along with the thread, or drop, into the medium which is to determine whether

the organisms do or do not possess the power of growth, and there have a restraining or antiseptic action. For organisms in their normal condition—that is, those which have never been exposed to the action of a disinfectant—the amount of certain disinfectants that is necessary to restrain growth is very small indeed; and for organisms that have already been exposed for a time to such agents this amount is very much less. It is plain, then, that if the test is to be an accurate one, precautions must be taken against admitting this minute trace of disinfectant to the medium with which we are to determine whether the bacteria exposed to the disinfectant were killed or not. The precautions hitherto taken to prevent this accident have been, when the threads were employed, washing them in sterilized distilled water and then in alcohol; or, where fluid cultures were mixed with the disinfectant in solution, an effort was usually made to dilute the amount of disinfectant carried over, to a point at which it lost its inhibiting power.

While such precautions are sufficient in many cases, they do not answer for all. Certain chemicals have the property of combining so firmly with the threads upon which the bacteria are located as to require other special means of ridding the threads of them; and in solutions in which proteid substances are present along with the bacteria a similar union between them and the disinfectant may likewise take place. In both instances this amount of disinfectant adhering to the threads or in combination with the proteids must be gotten rid of, otherwise the results of the test may be fallacious. A partial solution of the problem is given through studies that have been made upon corrosive sublimate in its various

applications for disinfecting purposes, and in this connection it has been shown by Shaefer[1] that it is impossible to rid silk threads of the corrosive sublimate adhering to them by simple washing, as the sublimate acts as a mordant and forms a firm union with the tissues of the threads. Braatz[2] found the same to hold good for catgut. For example, he found that catgut which had been immersed in solutions of corrosive sublimate gave the characteristic reactions of the salt after having been immersed for five weeks in distilled water which had been repeatedly renewed. Braatz remarks that a similar combination between sublimate and cotton will take place after a long time; but it occurs so slowly that it cannot interfere with disinfection-experiments in the same way that silk does.

The most successful attempt at removing all traces of sublimate from the threads or from the proteid substances in which are located the bacteria whose vitality is to be tested was made by Geppert, who subjected them to the action of ammonium sulphide in solution. By this procedure the mercury is converted into inert, insoluble sulphide, and has no inhibiting effect upon the growth of those bacteria that did not succumb to its action when in the form of the bichloride.

In the second method of testing disinfectants mentioned above—that is, when cultures of bacteria and solutions of the disinfectant are mixed, and after a time a drop of the mixture is removed and added to sterile nutrient media—the inhibiting amount of disinfectant can readily be gotten rid of by dilution; that is to say,

[1] Shaefer: Berliner klin. Wochenschrift, 1890, No. 3, p. 50.

[2] Braatz: Centralblatt für Bakteriolgie und Parasitenkunde, Bd. vii. No. 1, p. 8.

instead of transferring the drop directly to the fresh medium, add it to 10 or 12 c.c. of sterilized salt-solution (0.6–0.7 per cent. of NaCl in distilled water) or distilled water, and after thoroughly shaking add a drop of *this* to the medium in which the power of development of the bacteria is to be determined.

Another important point to be borne in mind in testing disinfectants is the necessity of so adjusting the conditions that each individual organism will be exposed to the action of the agent used. When clumps of bacteria exist we are not always assured of this, for only those on the surface of the clump may be affected, while those in the centre of the mass may escape, being protected by those surrounding them. These clumps and minute masses are especially liable to be present in fluid cultures and in suspensions of bacteria, and must be eliminated before the test is begun, if this is to be made by mixing them with solutions of the agent to be tested. This is best accomplished in the following way: the organisms should be cultivated in bouillon containing sand or finely divided particles of glass; after growing for a sufficient length of time they are to be shaken thoroughly, in order that all clumps may be mechanically broken up by the sand. The culture is then filtered through a tube containing closely packed glass-wool.

The filtration may be accomplished without fear of contamination of the culture by the employment of an Allihin tube, which is practically a thick-walled test-tube drawn out to a finer tube at its blunt end so as to convert it into a sort of cylindrical funnel. The tube when ready for use has the appearance shown in Fig. 109.

This tube, after being plugged at the bottom with glass-wool (*a*, Fig. 109), and at its wide extremity with cotton-wool, is placed vertically, small end down, into an Erlenmeyer flask of about 100 c.c. capacity and sterilized in a steam sterilizer for the proper time. It is kept in the sterilizer until it is to be used, which should be as soon as possible after sterilization.

FIG. 109.

Cylindrical funnel used for filtering cultures on which disinfectants are to be tested.

The watery suspension or bouillon culture of the organisms is now to be filtered repeatedly through the glass-wool into sterilized flasks until a degree of transparency is reached which will permit the reading of moderately fine print through a layer of the fluid about 2 cm. thick—*i. e.*, through an ordinary test-tube full of it. This filtrate can then be subjected to the action of the disinfectant. As a rule, the results are more uniform than when no attention is paid to the presence of clumps. It is scarcely necessary to say that in the practical employment of disinfectants outside the laboratory no such precautions are taken; but in laboratory work, where it is desired to determine *exactly* the value of different substances as germicides, all the precautions mentioned will be found essential to precision.

The disinfectant value of gases and vapors is determined by their action upon test-objects in closed

chambers. The object is to determine the proportion of the gas, when mixed with air, that is required to destroy the bacteria exposed to its action in a given time. For this purpose the test is usually made as follows: under a sterilized bell-glass of known capacity the test-objects are placed. Into the chamber is then admitted sufficient of a mixture of air and the gas under consideration, of known proportions, to displace completely all the air; or the pure gas itself may be introduced in amount necessary to give the desired dilution when mixed with the air in the chamber. At the expiration of the time decided upon for the test the infected articles are removed and the vitality of the bacteria upon them is determined.

In the case of vapors of volatile fluids, such, for instance, as formalin, the fluid is placed under the bell-glass in an open dish; in another open dish the test-objects are placed. The bell-glass is then sealed to an underlying ground-glass plate by vaselin or paraffin, and the fluid is allowed to vaporize at ordinary room-temperature. The point here to be decided is the volume or weight of such a fluid that it is necessary to expose in an air-chamber of known cubic capacity in order that bacteria may be destroyed by its vapor in a given time.

In determining the germicidal value of different chemical agents for certain pathogenic bacteria susceptible animals are sometimes inoculated with the organisms after they have been exposed to the disinfectant. If no pathological condition results, disinfection is presumed to have been successful; while if the condition characteristic of the activities of the given organism in the tissues of this animal appears, the

reverse is the case. The objections to this method are: "First. The test-organisms may be modified as regards reproductive activity without being killed; and in this case a modified form of the disease may result from the inoculation, of so mild a character as to escape observation. Second. An animal that has suffered this modified form of the disease enjoys protection, more or less perfect, from future attacks, and if used for a subsequent experiment may, by its immunity from the effects of the pathogenic test-organism, give rise to the mistaken assumption that this had been destroyed by the action of the germicidal agent to which it had been subjected." (Sternberg.)

DETERMINATION OF ANTISEPTIC PROPERTIES.

For this purpose sterile media are employed, and are usually arranged in two groups: the one to remain normal in composition and to serve as controls, while to the other the substance to be tested is to be added in different but known strengths. It is customary to employ test-tubes each containing an exact amount of bouillon, gelatin, or agar-agar, as the case may be. To each tube a definite amount of the antiseptic is added, and if it is not of a volatile nature or not injured by heat, the tubes may then be sterilized. After this they are to be inoculated with the organism with which the test is to be made, and at the same time one of the "control"-tubes (one of those to which no antiseptic has been added) is inoculated. They are all then to be placed in the incubator and kept under observation. If at the end of twenty-four, forty-eight, or seventy-two hours no growth appears in any but the "control"-tubes, it is evident that the antiseptic must be added in smaller

amounts, for we are to determine the point at which it *is not* as well as that at which it *is* capable of preventing development. The experiment is then repeated, using smaller amounts of the antiseptic until we reach a point at which growth just occurs, notwithstanding the presence of the antiseptic; the amount necessary for antisepsis is then a trifle greater than that used in the last tube. If, for example, there was no development in the tubes in which the antiseptic was present in the proportion of 1 : 1000, and growth in the one in which it was present in 1 : 1400, the experiment should be repeated with strengths of the antiseptic corresponding to 1 : 1000, 1 : 1100, 1 : 1200, 1 : 1300, 1 : 1400, and in this way one ultimately determines the amount by which growth is *just* prevented; this represents the antiseptic value of the substance for the organism with which it was tested.

EXPERIMENTS.

To each of three tubes containing 10 c.c.—one of physiological salt-solution, another of bouillon, a third of fluid blood-serum—add as much of a culture of *staphylococcus pyogenes aureus* as can be held upon a looped platinum wire. Break this up carefully to eliminate clumps, and then add exactly 10 c.c. of a 1 : 500 solution of corrosive sublimate. Mix thoroughly, and at the end of three minutes transfer a drop from each tube into tubes of liquefied agar-agar, and pour these into Petri dishes. Label each dish carefully and place them in the incubator. Are the results the same in all the plates? How are the differences to be explained? To what strength of the disinfectant were the organisms exposed in the experiment?

To each of two tubes—the one containing 10 c.c. of normal salt-solution, the other of bouillon—add as much of a spore-containing culture of anthrax bacilli as can be held upon a loop of platinum wire. Distribute this uniformly through the medium, and then add exactly 10 c.c. of a 1 : 500 solution of corrosive sublimate. Mix thoroughly, and at the end of five minutes transfer a drop from each tube to tubes of liquefied agar-agar. Pour these immediately into Petri dishes. Label each dish carefully and place them in the incubator. Note the results at the end of twenty-four, forty-eight, and seventy-two hours. How do you explain them?

Make identically the same experiment with the same spore-containing culture of anthrax bacilli, except that the drop from the mixture is to be transferred to 10 c.c. of a mixture of equal parts of ammonium sulphide and sterilized distilled water. After remaining in this for about half a minute, a drop is to be transferred to a tube of liquefied agar-agar, poured into Petri dishes, labelled, and placed in the incubator. Note the results. Do they correspond with those obtained in the preceding experiment? How are the differences explained?

Prepare a 1 : 1000 solution of corrosive sublimate. To each of twelve tubes containing exactly 10 c.c. of bouillon add one drop to the first, two drops to the second, and so on until the last tube has had twelve drops added to it. Mix thoroughly and then inoculate each with one wire-loopful of a bouillon culture of *staphylococcus pyogenes aureus*. Place them all in the

incubator after carefully labelling them. Note the order in which growth appears.

Do the same with anthrax spores, with spores of *bacillus subtilis*, and with the typhoid bacillus, and compare the results. From these experiments, what will be the strength of corrosive sublimate necessary to antisepsis under these conditions for the organisms employed?

Make a similar series of experiments using a 5 per cent. solution of carbolic acid.

Determine the antiseptic value of the common disinfectants for the organisms with which you are working.

Determine the time necessary for the destruction of the organisms with which you are working, by corrosive sublimate in 1 : 1000 solution, under different conditions—with and without the presence of albuminous bodies other than the bacteria, and under varying conditions of temperature.

In making these experiments be careful to guard against the introduction of sufficient sublimate into the agar-agar with which the Petri plate is to be made to inhibit the growth of the organisms which may not have been destroyed by the sublimate. This may be done by transferring two drops from the mixture of sublimate and organism into not less than 10 c.c. of sterilized physiological salt-solution, in which they may be thoroughly shaken for from one to two minutes, or into the solution of ammonium sulphide of the strength given.

To 10 c.c. of a bouillon culture of *staphylococcus pyogenes aureus* or anthrax spores add 10 c.c. of a

1 : 500 solution of corrosive sublimate, and allow it to remain in contact with the organisms for *only one-half* the time necessary to destroy them (use an organism for which this has been determined). Then transfer a drop of the mixture to each of three liquefied agar-agar tubes and pour them into Petri dishes. Place them in the incubator and observe them for twenty-four, forty-eight, and seventy-two hours. No growth occurs. How is this to be accounted for?

At the end of seventy-two hours inoculate all of these plates with a culture of the same organism which has *not been exposed* to sublimate, by taking up bits of culture on a needle and drawing it across the plates. A growth now results. We have here an experiment in which organisms which have been exposed to sublimate for a *much shorter time* than necessary to destroy them, when transferred directly to a favorable culture-medium do not grow, and yet, when the same organism which has not been exposed to sublimate at all is planted upon the same medium it does grow. How is this to be accounted for?

SKIN-DISINFECTION.—With a sterilized knife scrape from the skin of the hands, at the root of the nails, and under the nails, small particles of epidermis. Prepare plates from them. Note the results.

Wash the hands carefully for ten minutes in hot water and scrub them during this time with soap and a sterilized brush. Rinse them in hot water. Again prepare plates from scrapings of the skin on the fingers, at the root of the nails, and under the nails. Note the results.

Again wash as before in hot water with soap and

brush, rinse in hot water, then soak the hands for five minutes in 1 : 1000 corrosive sublimate solution, and, as before, prepare plates from scrapings from the same localities. Note the results.

Repeat this latter procedure in exactly the same way, but before taking the scrapings let some one pour ammonium sulphide over the points from which the scrapings are to be made. After it has been on the hands about three minutes again scrape, and note the result upon plates made from the scrapings.

Wash as before in hot water and soap, rinse in clean hot water, immerse for a minute or two in alcohol, after this in 1 : 1000 sublimate solution, and finally in ammonium sulphide, and then prepare plates from scrapings from the points mentioned.

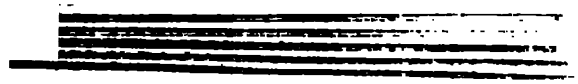
In what way do the results of these experiments differ from one another?

To what are these differences due?

What have these experiments taught?

In making the above experiments it must be remembered that the strictest care is necessary in order to prevent the access of germs from without into our media. The hand upon which the experiment is being performed must be held away from the body and must not touch any object not concerned in the experiment. The scraping should be done with the point of a knife that has been sterilized in a flame and allowed to cool. The scrapings may be transferred directly from the knife-point to the gelatin by means of a sterilized platinum wire loop.

The brush used should be thoroughly cleansed and always kept in 1 : 1000 solution of corrosive sublimate. It should be washed in hot water before using.



APPENDIX.

LIST of apparatus and materials required in a beginner's bacteriological laboratory:

MICROSCOPE AND ACCESSORIES.

Microscope with coarse and fine adjustment and heavy, firm base; Abbe sub-stage condensing system, arranged either as the "simple" or as the regular Abbe condenser, in either case to be provided with iris diaphragm; objectives equivalent, in the English nomenclature, to about one-fourth inch and one-sixth inch dry, and one-twelfth inch oil-immersion system; a triple revolving nose-piece; three oculars, varying in magnifying power; and a bottle of immersion oil.

Glass slides, English shape and size and of colorless glass.

Six slides with depressions of about 1 cm. in diameter in centre.

Cover-slips, 15 by 15 mm. square and not more than from 0.15 to 0.18 mm. thick.

Forceps. One pair of fine-pointed forceps and one pair of the Cornet or Stewart pattern, for holding cover-slips.

Platinum needles with glass handles. One straight, about 4 cm. long; one looped at the end, about 4 cm. long; and one straight, about 8 cm. long. Glass

handles to be about 3 mm. in thickness and from 15 to 17 cm. long.

STAINING- AND MOUNTING-REAGENTS.

200 c.c. of saturated alcoholic solution of fuchsin.

200 c.c. of saturated alcoholic solution of gentian-violet.

200 c.c. of saturated alcoholic solution of methylene-blue.

200 grammes of pure aniline.

200 grammes of C. P. carbolic acid.

500 grammes of C. P. nitric acid.

500 grammes of C. P. sulphuric acid.

200 grammes of C. P. glacial acetic acid.

1 litre of ordinary 93–95 per cent. alcohol.

1 litre of absolute alcohol.

500 grammes of ether.

500 grammes of pure xylol.

50 grammes of Canada balsam dissolved in xylol.

100 grammes of Schering's celloidin.

10 grammes of iodine and 30 grammes of potassium iodide in substance.

100 grammes of tannic acid.

100 grammes of ferrous sulphate.

Distilled water.

FOR NUTRIENT MEDIA.

¼ pound of Liebig's or Armour's beef-extract.

250 grammes of Witte's or Sargent's peptone.

2 kilogrammes of gold-label gelatin (Hesteberg's).

100 grammes of agar-agar in substance.

200 grammes of sodium chloride (ordinary table-salt).

500 grammes of pure glycerin.

50 grammes of pure glucose.
20 grammes of pure lactose.
100 grammes of caustic potash.
200 c.c. of litmus tincture.
10 grammes of rosolic acid (corallin).
Blue and red litmus-paper; curcuma paper.
5 grammes of phenolphtalein in substance.
Filter-paper, the quality ordinarily used by druggists.
100 grammes of pyrogallic acid.
1 kilogramme of C. P. granulated zinc.

GLASSWARE.

200 best quality test-tubes, slightly heavier than those used for chemical work, about 12 to 13 cm. long and 12 to 14 mm. inside diameter.

15 Petri double dishes about 8 or 9 cm. in diameter and from 1 to 1.5 cm. deep.

6 Florence flasks, Bohemian glass, 1000 c.c. capacity.

6 Florence flasks, Bohemian glass, 500 c.c. capacity.

12 Erlenmeyer flasks, Bohemian glass, 100 c.c. capacity.

1 graduated measuring-cylinder, 1000 c.c. capacity.

1 graduated measuring-cylinder, 100 c.c. capacity.

25 bottles, 125 c.c. capacity, narrow necks with ground-glass stoppers.

25 bottles, 125 c.c. capacity, wide mouths, with ground-glass stoppers.

1 anatomical or preserving jar, with tightly fitting cover, of about 4 litres capacity, for collecting blood-serum.

2 battery jars of about 2 litres capacity, provided with loosely fitting, weighted, wire-net covers for mice.

10 feet of soft-glass tubing, 2 or 3 mm. inside diameter.

20 feet of soft-glass tubing, 4 mm. inside diameter.

6 glass rods, 18 to 20 cm. long and 3 or 4 mm. in diameter.

6 pipettes of 1 c.c. each, divided into tenths.

2 pipettes of 10 c.c. each, divided into cubic centimetres and fractions.

1 burette of 50 c.c. capacity, divided into cubic centimetres and fractions.

1 separating-funnel of 750 c.c. capacity for filling tubes.

2 glass funnels, best quality, about 15 cm. in diameter.

2 glass funnels, best quality, about 8 cm. in diameter.

2 glass funnels, best quality, about 4 or 5 cm. in diameter.

2 porcelain dishes, 200 c.c. capacity.

6 ordinary water tumblers for holding test-tubes.

1 ruled plate for counting colonies.

1 gas-generator, 600 c.c. capacity, pattern of Kipp or v. Wartha.

BURNERS, TUBING, ETC.

2 Bunsen burners, single flame.

1 Rose-burner.

1 Koch safety-burner, single flame.

6 feet of white-rubber gas-tubing.

12 feet of pure red-rubber tubing, 5 to 6 mm. inside diameter.

1 thermo-regulator, pattern of L. Meyer or Reichert.

2 thermometers, graduated in degrees of Centigrade, registering from 0 to 100° C., graduated on the stem.

1 thermometer graduated in tenths and registering from 0 to 50° C.

1 thermometer registering to 200° C.

INSTRUMENTS, ETC.

1 microtome, pattern of Schanze, with knife.

1 razor-strop.

6 cheap-quality scalpels, assorted sizes.

2 pairs heavy dissecting-forceps.

1 pair medium-size straight scissors.

1 pair small-size straight scissors.

1 hypodermic syringe that will stand steam sterilization.

2 teasing-needles.

1 pair long-handled crucible-tongs for holding mice.

1 wire mouse-holder.

2 small pine boards on which to tack animals for autopsy.

2 covered stone jars for disinfectants and for receiving infected materials.

INCUBATORS AND STERILIZERS.

1 incubator, simple square form, either entirely of copper or of galvanized iron with copper bottom.

1 medium-size hot-air sterilizer with double walls, asbestos jacket, and movable false bottom of copper plates.

1 medium-size steam sterilizer; either the pattern of Koch or that known as the Arnold steam sterilizer, preferably the latter.

MISCELLANEOUS.

1 pair of balances, capacity 1 kilogramme; accurate to 0.2 gramme.

1 set of cork-borers.

1 hand-lens.

1 wooden filter-stand.

2 iron stands with rings and clamps.

3 round, galvanized iron-wire baskets to fit loosely into steam sterilizer.

3 square, galvanized iron-wire baskets to fit loosely into hot-air sterilizer.

1 sheet-iron box for sterilizing pipettes, etc.

1 covered agate-ware saucepan, 1200 c.c. capacity.

2 iron tripods.

1 yard of moderately heavy wire gauze.

2 test-tube racks, each holding 24 tubes, 12 in a row.

1 constant-level, cast-iron water-bath.

2 potato-knives.

2 test-tube brushes with reed or wire handles.

Cotton-batting.

Copper wire, wire nippers.

Round and triangular files.

Labels.

Towels and sponges.

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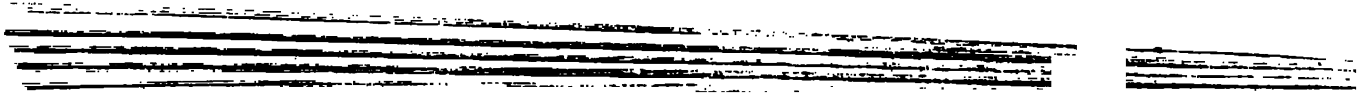

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